Cytosolic GLUTAMINE SYNTHETASE1;1 Modulates Metabolism and Chloroplast Development in Roots

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Nitrogen (N) is an essential macronutrient, and the final form of endogenous inorganic N is ammonium, which is assimilated by Gln synthetase (GS) into Gln. However, how the multiple isoforms of cytosolic GSs contribute to metabolic systems via the regulation of ammonium assimilation remains unclear. In this study, we compared the effects of two rice (Oryza sativa) cytosolic GSs, namely OsGS1;1 and OsGS1;2, on central metabolism in roots using reverse genetics, metabolomic and transcriptomic profiling, and network analyses. We observed (1) abnormal sugar and organic N accumulation and (2) significant up-regulation of genes associated with photosynthesis and chlorophyll biosynthesis in the roots of Osgs1;1 but not Osgs1;2 knockout mutants. Network analysis of the Osgs1;1 mutant suggested that metabolism of Gln was coordinated with the metabolic modules of sugar metabolism, tricarboxylic acid cycle, and carbon fixation. Transcript profiling of Osgs1;1 mutant roots revealed that expression of the rice sigma-factor (OsSIG) genes in the mutants was transiently upregulated. GOLDEN2-LIKE transcription factor-encoding genes, which are involved in chloroplast biogenesis in rice, could not compensate for the lack of OsSIGs in the Osgs1;1 mutant. Microscopic analysis revealed mature chloroplast development in Osgs1;1 roots but not in the roots of OsGs1;2, OsGs1;2-complemented lines, or the wild type. Thus, organic N assimilated by OsGS1;1 affects a broad range of metabolites and transcripts involved in maintaining metabolic homeostasis and plastid development in rice roots, whereas OsGS1;2 has a more specific role, affecting mainly amino acid homeostasis but not carbon metabolism.

Increasing the rate or efficiency of nitrogen (N) assimilation is essential for achieving sustainable agriculture. N assimilation is a crucial step in the synthesis of organic N from the inorganic forms available in the soil. Like carbon (C), N is a vital macronutrient essential for efficient plant growth and crop productivity. N is required by plants to produce N-containing molecules, including chlorophylls, amino acids, and proteins, including enzymes (Stitt and Fernie, 2003; Kusano et al., 2011a; Fontaine et al., 2012; Wang et al., 2014). Therefore, it is necessary to understand the regulatory mechanisms that control the primary steps of inorganic N assimilation and the subsequent biochemical pathways involved in N and C metabolism.

Rice (Oryza sativa) is one of the most important crops in the world (http://www.fao.org/docrep/006/Y4751E/y4751e05.htm). Paddy rice is adapted to waterlogged and reductive soil and preferentially uses ammonium (via Gln synthetase [EC. 6.3.1.2] [GS]) instead of nitrate, whereas most nonwetland plant species use nitrate (via nitrate reductase) as their inorganic N source. Based on molecular mass, quaternary protein structure, and genome sequences, there are two types of GS (GS1 and GS2) encoded by a multigene family in eukaryotes (García-Domínguez et al., 1997; Eisenberg et al., 2000; Ghoshroy et al., 2010; García-Calderón et al., 2012). Higher plants possess GS1, localized in the cytosol, and GS2, localized in the plastids. Three to 16 genes encode cytosolic GS in plants. For example, there are three homologous GS1 genes in rice (Ishiyama et al., 2004a), barley (Hordeum vulgare; Goodall et al., 2013), and wheat (Triticum aestivum; Wang et al., 2015), two in Medicago truncatula (Carvalho et al., 2000), five each in maize (Zea mays; Li et al., 1993; Martin et al., 2006) and Arabidopsis (Arabidopsis thaliana; Ishiyama et al., 2004b), and 16 in Brassica napus (Orsel et al., 2014). However, it remains unclear why plants have multiple isoforms of GS1 and what their different functions might be. To address this issue, the specific functions of
cytosolic GS in terms of C/N balance, N uptake, yield, stress response, and senescence in plants have been characterized (Watanabe et al., 1994; Fei et al., 2006; Lothier et al., 2011; Lu et al., 2011; Molina-Rueda et al., 2013; Seifi et al., 2013; Guan et al., 2015).

In rice tissues, the three GS1 genes (OsGS1;1, OsGS1;2, and OsGS1;3) are expressed in distinctive patterns (Tabuchi et al., 2005). OsGS1;1 mRNA is detected in shoots and roots, whereas transcripts of OsGS1;2 and OsGS1;3 are present in roots and spikelets, respectively. The loss of OsGS1;1 and OsGS1;2, as a result of loss-of-function knockout mutations has negative impacts on shoot growth and yields in the presence of adequate ammonium (Tabuchi et al., 2005; Kusano et al., 2011b; Funayama et al., 2013; Ohashi et al., 2015). These findings suggest that there is a yet-to-be identified link to explain how and why the mutants could achieve such different and specific responses in rice.

Gene expression data in published articles have been accumulated in repositories, including NCBI Gene Expression Omnibus (GEO; Barrett et al., 2013) and ArrayExpress (Kolesnikov et al., 2015). Gene network analysis based on the deposited data facilitates the identification of gene network clusters that are changed by nutrients, such as N (Gutiérrez et al., 2007; Varala et al., 2018; Brooks et al., 2019), and by abiotic/biologic stresses in plants (Kilian et al., 2007; Lee et al., 2010, 2011; Kusano et al., 2011c; Dash et al., 2018). The visualization of gene networks is crucial to depict the network topology and to enable a differential coexpression analysis and the accurate interpretation of data. On the other hand, network analysis by integrating the profiles of metabolites and enzyme-coding genes can identify candidates of active metabolic subnetworks based on metabolite-enzyme reactions obtained from a publicly available database, such as Kyoto Encyclopedia of Genes and Genomes (KEGG; Sereushichev et al., 2016; Hiemer et al., 2019).

In this study, we first aimed to determine clear differences or similarities between OsGS1;1 and OsGS1;2 in terms of their genome sequences, including their amino acid sequences and the distribution of short sequences on each promoter site. Our results demonstrate the critical roles of OsGS1;1 in achieving metabolic balance and plastid differentiation in rice roots. The study also indicates that OsGS1;2 is not able to compensate for the loss of these OsGS1;1 functions.

RESULTS

Comparison of Amino Acid Sequences and Promoter Regions between the Two OsGS1 Genes

It has been reported that the structures of the two rice GS1 genes (i.e. OsGS1;1 and OsGS1;2) are different (Tabuchi et al., 2005; Funayama et al., 2013). To characterize GS1 genes in rice, we constructed an alignment and a phylogenetic tree of representative GS1 genes from rice, Arabidopsis, maize, and soybean (Glycine max). In multiple alignments, highly conserved residues are colored to allow us to easily recognize highly conserved residues. GS1 genes in rice, Arabidopsis, maize, and soybeans were highly conserved (Supplemental Fig. S1). OsGS1;1 was highly similar to GRMZM2G036464 (ZmGln5, GS1-4) and GRMZM5G872068 (ZmGln4, GS1-3) in maize and formed a single group. By contrast, OsGS1;2 showed high similarity to GRMZM2G050514 (ZmGln6, GS1-1; Supplemental Fig. S2).

We then compared the local distribution of octamer sequences in the promoters of OsGS1;1 and OsGS1;2 (Yamamoto et al., 2007; Hieno et al., 2014). Promoter sequences of OsGS1;1 and OsGS1;2 showed very low similarity, which prevented us from generating a sequence alignment. In addition, the mapping of putative regulatory elements in these promoters did not detect any shared elements. These results suggest that these two genes are likely independently regulated (Supplemental Fig. S3).

1This research was supported by JST PRESTO (JPMJPR13B5 to M.Ku.) and a Grant-in-Aid for Scientific Research on Innovative Areas (22119003 to T.Y.) from the Ministry of Education, Culture, Sports, Science, and Technology of Japan. This work was partly supported by JSPS KAKENHI (grant no. 17K07663 to A.F.). This research was also supported by the “Sustainable Food Security Research Project” in the form of an operational grant from the National University Corporation, Japan.

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M.Ku., K.F., K.M., A.F., K.S., and T.Y. designed the research; M.Ku., K.M., K.F., M.T.-K., S.K., T.N., M.Ko., and C.F. prepared samples and performed research; M.W., M.Sa., and K.T. conducted TEM analysis; M.Ku. and K.O.-K. conducted PAM analysis; Y.U. and M.Se. conducted starch quantification; M.Ku., A.F., K.M., and Y.Y.Y. analyzed data; and M.Ku. and T.Y. wrote the paper.

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www.plantphysiol.org/cgi/doi/10.1104/pp.19.01118
Prediction of OsGS1;1 and OsGS1;2 Functions by Combining Five Gene Network Inference Methods Using Publicly Available Data

We used the publicly available transcript profiles of rice seedlings and root samples to conduct a gene network analysis by integrating five gene network inference methods and identifying genes associated with each cytosolic GS1 in rice (Supplemental Datasets S1 and S2). After integrating the predictions for each OsGS1 gene, we chose gene pairs that were common across the individual methods to construct gene networks. There were 49 gene pairs for OsGS1;1 and 17 pairs for OsGS1;2 in shoot datasets, whereas there were 142 gene pairs for OsGS1;1 and 220 pairs for OsGS1;2 in root datasets.

Gene ontology (GO) term enrichment analysis was conducted to visualize each gene network. Though we could not construct gene networks using the results of the GO term enrichment analysis of shoot datasets, the enrichment analysis of root datasets revealed many more connections between the GO categories in the OsGS1;1 gene network (130 nodes and 202 edges; Supplemental Fig. S4A) than in the OsGS1;2 gene network (31 nodes and 34 edges; Supplemental Fig. S4B). Of the 65 GO categories that were significantly overrepresented in the OsGS1;1 gene network, cytosolic 6-phosphogluconate dehydrogenase1 (6PGDH1) in the oxidative pentose phosphate pathway, glycerol-3-phosphate dehydrogenase (GPDH) as a component of the glycerol-3-phosphate shuttle (Shen et al., 2006), and NADP-specific isocitrate dehydrogenase (IDH) in the tricarboxylic acid cycle were correlated with OsGS1;1 in the cellular carbohydrate metabolic process categories. Conversely, among the 14 GO categories, the ion transport category, which contains four transporter genes and three ion channel/pump genes, were overrepresented in the OsGS1;2 gene network. There was no overlap between the genes associated with OsGS1;1 and OsGS1;2 (Supplemental Fig. S4C). These results imply that, compared with OsGS1;2 gene pairs, OsGS1;1 and genes involving N- and C-metabolism are more closely associated.

Visible Phenotypes of the Two Osgs1 Mutants during the Third- and Fourth-Leaf Seedling Stages

We conducted a reverse-genetics approach using mutants lacking Osgs1;1 and Osgs1;2 to determine the function of these two GS1 enzymes in the presence of ammonium ions as a sole N source. As suggested by the comparison of Osgs1;1 and Osgs1;2, mutant plants exhibited morphological changes at the grain-filling and mature stages (Tabuchi et al., 2005; Funayama et al., 2013). No marked seedling phenotypic differences were observed at the third-leaf stage in the Osgs1 mutant (Fig. 1; Supplemental Table S1) in the presence of adequate ammonium, as previously described by Kusano et al. (2011b). After the fourth-leaf stage, the above-ground portion of Osgs1;1 mutant seedlings exhibited a 50% reduction in growth compared to the control seedlings (Fig. 1; Supplemental Fig. S5), but this effect was not observed in Osgs1;2 seedlings (Fig. 1). To minimize any effects associated with these visible phenotypic differences between the Osgs1 mutants and the control samples, the growth period at the third-leaf stage was chosen for further analyses (Supplemental Fig. S6).

Carbohydrate Metabolism Is Closely Coordinated in Roots of the Osgs1;1 Mutant but Not in Roots of the Osgs1;2 Mutant

To investigate the metabolite composition of Osgs1;1 and Osgs1;2 mutants, gas chromatography-mass spectrometry was employed for metabolite profiling of the third-leaf stage leaf blade (LB), the third-leaf stage leaf sheath, and root samples of each of the two Osgs1 mutants, the nullizygous (null) lines from Osgs1 mutants, and wild type grown in hydroponic cultures with 1.0 mM of ammonium as the sole N source (Supplemental Materials and Methods). We detected 225 peaks from this profiling. Among these, 82 peaks were identified using the information obtained by mass spectra matching and the retention index of compounds in mass spectral libraries or through comparison to authentic standards. The identified metabolites in the profile data contained 13 carbohydrates (including sugars and sugar phosphates), 26 amino acids, 21 organic acids, and five amines (Supplemental Dataset S3).

We conducted a principal component analysis (PCA) of metabolite profile data to identify general trends of sample distributions. The PCA score scatter plot revealed that samples were clustered according to genotype-dependent trends among the controls (wild-type and null lines) and the Osgs1;1 and Osgs1;2 mutants for each tissue (Supplemental Fig. S7). In the profiles of the three tissues of the Osgs1;1 mutant, there were significant increases in the levels of sugars and sugar phosphates (Fig. 2; Supplemental Fig. S8; Supplemental Dataset S3). Additionally, amino acid levels increased in the profiles of Osgs1;1 mutant roots. By contrast, the profiles of LB, leaf sheath, and the roots of the Osgs1;2 mutants exhibited a significant decrease in the levels of amino acids and polyamines. The comparison of metabolite changes in each of the three tissues of Osgs1;1 and Osgs1;2 mutants and their importance in central metabolism is summarized in Supplemental Figure S8.

Because Osgs1;2 is mainly expressed in the roots, we focused on the root metabolite profiles for comparing differences between Osgs1;1 and Osgs1;2 (Tabuchi et al., 2005). In the Osgs1;1 mutant, a significant increase in amino acid content associated with the Glu and Asp pathways was observed in the roots. In these pathways, Gln and Asn are produced from ammonium and catalyzed by GS and Asn synthetase, respectively.
Relationships between Gln and Asn were investigated to obtain insights into the metabolic balance in OsGS1;1, OsGS1;2, and the corresponding controls. Correlation analysis between the levels of Gln and Asn in the OsGS1;1 mutant in the roots showed close coordination, whereas such coordination was absent from the OsGS1;2 mutant and the controls (Supplemental Table S2). Carbohydrate levels such as Suc and trehalose increased.

| Amino acids       | OsGS1;1 vs. null | OsGS1;2 vs. WT | Carbohydrates          | OsGS1;1 vs. null | OsGS1;2 vs. WT |
|-------------------|------------------|----------------|------------------------|-----------------|----------------|
| 1,3-DAP           | 2.5              | -0.3           | T6P                    | 3.9             | 0.4            |
| Glutamine         | 2.3              | -3.3           | Trehalose              | 2               | 0.3            |
| Ornithine         | 2.1              | -3             | Sucrose                | 1.7             | -0.2           |
| Pyroglutamate     | 2.1              | -2.8           | Ribitol                | 1.2             | -0.8           |
| Glutamate         | 2                | -1.4           | Ribose                 | 0.8             | -3             |
| 3-CA              | 1.9              | -2.8           |                        |                 |                |
| Cysteine          | 1.9              | -5             | Isocitrate             | 2.8             | -3.2           |
| Asparagine        | 1.8              | -4.1           | Citrate                | 1.3             | -1.9           |
| Alanine           | 1.7              | -1.9           | Phosphate              | 1.1             | 0.1            |
| Threonine         | 1.7              | -0.6           | Aconitate              | -0.4            | -1.6           |
| Phenylalanine     | 1.6              | -2.1           |                        |                 |                |
| Aspartate         | 1.5              | -1.5           | Suberate               | 2               | -2.9           |
| Proline           | 1.3              | -1.5           | Shikimate              | 1.5             | -0.2           |
| Isoleucine        | 1.2              | 0.2            | Threonate              | 0.4             | -1.3           |
| Valine            | 1.2              | -0.6           | Dihydouracil           | 1.3             | -0.6           |
| Serine            | 1.1              | -0.6           | Glycerol               | 0.9             | 0              |
| GABA              | 0.9              | 1.4            |                        |                 |                |

**Polyamines**

| Spermidine | 0.3       |                              | Putrescine | -0.8     | 2        |
Transcript Abundances of Photosynthesis-Related Genes Are Increased in the Roots of Two Osgs1;1-Knockout Mutants at the Third-Leaf Stage

To investigate whether the presence of organic N assimilated by OsGS1;1 or OsGS1;2 affects transcript abundances in rice roots, we performed transcript profiling of Osgs1-knockout mutants and their corresponding controls. A Venn diagram was used to represent the number of transcripts that were commonly or specifically changed in the Osgs1;1 and Osgs1;2 profiles (Supplemental Fig. S9). Less than 2% of transcripts were shared between these Osgs1 mutant profiles. Next, the transcript profile data were overlaid in MapMan (Usadel et al., 2005) to visualize overrepresented MapMan bin codes (Fig. 4; Supplemental Figs. S10–S12). We found that genes encoding the components of PSI, electron carriers, cytochrome b6/f, PSI, and ATPase in the chloroplast were upregulated in the roots of the Osgs1;1 mutant. By contrast, we observed no significant changes in the expression levels of photosynthesis-related genes in the roots of the Osgs1;2 mutant. Of the genes exhibiting significantly altered gene expression in the profiles of two independent knockout mutants of Osgs1;1 (NC2373 and ND8037), 108 genes were commonly upregulated, whereas 68 genes were downregulated. Regarding photosynthesis-related genes, 34 genes common to both allelic variants were upregulated, including genes involved in the light reaction, Calvin cycle, and tetrapyrrole synthesis (Supplemental Dataset S5). To verify the results of the microarray data, we performed reverse transcription quantitative PCR (RT-qPCR) assays to quantify the mRNA content of 10 representative photosynthesis-related genes encoding ferredoxin-NADP reductase (FRD), PSI reaction center subunit N (PN), glutamyl-tRNA reductase (HEMA1), magnesium-protoporphyrin IX monomethyl ester cyclase (CRD1), and Fru-1,6-bisphosphatase (FBPase) in the mutant roots (Fig. 4). We also quantified mRNA abundances of genes OsLhcb1 and OsLhcb2, which encode proteins of the light-harvesting complex of PSI in the chloroplast, and found that transcript abundances of the genes were also increased in both mutants relative to the control. To investigate whether exogenous Suc addition affects expression levels of photosynthesis-related genes, we conducted RT-qPCR analysis of photosynthesis-related genes (Supplemental Tables S3 and S4). There were no significant changes in the transcript levels of these genes following Suc treatment, except for the levels of OsHEMA1 and OsCRD.

The Osgs1;1 mutants also showed significant increases in the transcript abundances of photosynthesis-related genes at the third-leaf stage, i.e. part of the heterotrophic growth period (Supplemental Fig. S6). However, it remains unclear whether such up-regulation of photosynthesis-related genes was observed at the fourth-leaf stage (autotrophic growth stage). To obtain insights into this phenomenon, transcript profiling of the roots of the Osgs1;1 mutants was performed at the third- and fourth-leaf stages (Supplemental Fig. S13; Supplemental Tables S5 and S6). Photosynthesis-related genes were upregulated at the third-leaf stage, whereas they were not upregulated at the fourth-leaf stage.

Figure 3. Sugar and starch accumulation in Osgs1 mutants. A, The log2 FC of sugars in roots of two independent Osgs1;1 mutants, one Osgs1;2 mutant, Osgs1;2-complemented lines, and the corresponding controls. Number of biological replicates, n = 6 except for the null line, for which n = 12. *FDR < 0.05. **FDR < 0.005. ***FDR < 0.0005. B, Starch quantification in Osgs1;1 mutant and Osgs1;2 mutant roots, and the corresponding controls. Number of biological replicates, n = 3. Welch’s t test, *P < 0.05. We used mutant NC 2373 for starch quantification in Osgs1;1 mutant. Bar presents SD. Tre, Trehalose; NC, homozygous Osgs1;1 mutant allele NC2373; ND, homozygous Osgs1;1 mutant allele ND8037; Osgs1;2 Comp, Osgs1;2-complemented line; and null, nullizygous line segregated from heterozygous Osgs1;1 mutants; WT, wild type.
Figure 4. Transcript abundance changes in photosynthesis-related genes in root samples of Osgs1;1 and Osgs1;2 mutants. A, Overview of transcript abundance changes in genes categorized as “chloroplast” determined in root samples of Osgs1;1 and Osgs1;2 visualized by MapMan. Colored bar represents log2 FC of each probe set on Affymetrix GeneChip rice genome arrays (red, upregulated; blue, downregulated). B, Two independent Osgs1;1 mutants (NC2373, NC; and ND8037, ND) were used to determine the mRNA abundance of genes related to photosynthesis and chlorophyll biosynthesis in roots of the Osgs1;1 mutants and null plants segregated from heterozygous Osgs1;1 lines. mRNA abundance analyzed by RT-qPCR was normalized relative to actin mRNA in each tissue, and then log2 transformed. Values are the means of three independent biological replicates, except for mutant NC2373 (n = 2). Error bars indicate SD. Significant, P value by Welch’s t test < 0.05. *P value < 0.05; **P value < 0.005; ***P value < 0.0005.
The transcript abundances of four out of six sigma-factor (OsSIG) genes (OsSIG1, OsSIG2b, OsSIG3, and OsSIG6) exhibited expression patterns similar to those of the photosynthesis-related genes. This finding suggests that the increased expression of photosynthesis-related genes, including OsSIGs, represents transient expression at the third-leaf stage. By contrast, there were no marked expression changes observed for the two genes encoding GOLDEN2-LIKE1 and GOLDEN2-LIKE2 transcription factors (OsGLK1 and OsGLK2) during the seedling stages (Fig. 5). In addition, transcript abundances of GATA NITRATE-INDUCIBLE CARBON-METABOLISM-INVOLVED (GNC) and CYTOKININ-RESPONSIVE GATA1 (CGA1) genes showed no significant changes between mutant roots and wild-type roots (Supplemental Table S7). These genes, like SIGs in plants, are involved in chloroplast biogenesis (Rossini et al., 2001; Kasai et al., 2004; Hanoka et al., 2005; Lysenko, 2007; Tozawa et al., 2007; Nakamura et al., 2009; Waters et al., 2009; Powell et al., 2012; Wang et al., 2013; Chi et al., 2015).

Integrated Network Analysis of Osgs1;1 Data Reveals Key Subnetwork Modules in Central Metabolism

The metabolite and transcript profiling of the Osgs1;1 mutant revealed abnormal accumulation of sugars and amino acids and the up-regulation of photosynthesis-related genes (Figs. 2–4). Gene set enrichment analysis (GSEA) and metabolite set enrichment analysis (MSEA) were applied to obtain trends as to which biological terms were overrepresented in the transcript and metabolite changes in the Osgs1;1 mutants at the third-leaf stage (Supplemental Figs. S15–S17). The results from GSEA suggest that photosynthesis and related terms were overrepresented among the upregulated genes of the mutant profiles, compared to the control profile (Supplemental Fig. S15). This finding was consistent with the results of the MapMan visualization (Fig. 4; Supplemental Figs. S10–S12). MSEA revealed that terms of N-related metabolism, including “urea cycle,” “ammonia recycling,” and “glutamate metabolism,” were highlighted in the profiles of the increased metabolite changes (Supplemental Fig. S17).

To visualize the impact of the loss-of-function of OsGS1;1 on central metabolism in rice roots, we conducted an unbiased network analysis by integrating metabolite and transcript profiles using GAM (“genes and metabolites”) analysis (Sergushichev et al., 2016). GAM analysis of Osgs1;1 profiles highlighted four specific metabolic modules as subnetworks in KEGG pathways (Fig. 6; Supplemental Tables S8 and S9; Lancien et al., 2000). In module 1 (M1), Suc, trehalose 6-phosphate, and trehalose accumulated, whereas genes encoding enzymes related to sugar and starch metabolism showed significant down-regulation when compared to the control. M2 contained the subnetwork involving Asp and the tricarboxylic acid cycle. By contrast, there was significant up-regulation of genes belonging to the Calvin-Benson cycle in plants in M3. The M4 highlighted Trp production as a result of enzymatic conversion using Ser and indole-3-glycerol phosphate as substrates.
Unexpected Chloroplast Development in the Cortex Regions of Osgs1;1 Mutant Roots

Because the root tissue of the Osgs1;1 mutants, but not that of the Osgs1;2 mutants, exhibited significant up-regulation of photosynthesis-related genes at the third-leaf stage, we performed a transmission electron microscopy (TEM) analysis of roots of the Osgs1;1 mutant, the Osgs1;2 mutant, the Osgs1;1-complemented line, and wild type to determine whether chloroplast development was induced in Osgs1;1 roots. In cross sections of the maturation zone (MZ) V of Osgs1;1 mutant roots (http://ricexpro.dna.affrc.go.jp/; Sato et al., 2013), we investigated three regions consisting of distinct tissues (i.e. the epidermis, exodermis, and sclerenchyma), the cortex containing aerenchyma (cortex), and the endodermis, pericycle, and stele (Fig. 7; Takehisa et al., 2012). In the Osgs1;1 mutant, developed chloroplasts containing thylakoid membranes with grana stacks were present in the cortex but not in the endodermis/pericycle/stele regions of roots at the MZ V zone (Fig. 7). The development of such mature chloroplasts was also observed in the cortex region of independent loss-of-function mutants of Osgs1;1 at MZ III (Supplemental Fig. S18). No chloroplast development was observed in roots of the Osgs1;2 mutant, the Osgs1;2-complemented line, or wild type (Fig. 7; Supplemental Figs. S19 and S20).

To determine whether photosynthetic activities were expressed in the adventitious chloroplasts in Osgs1;1 roots, we analyzed chlorophyll fluorescence using a high-performance pulse-amplitude-modulation (PAM) chlorophyll fluorometer (Fig. 7; Supplemental Fig. S21; Supplemental Table S10). The effective quantum yield of PSII (ΦPI) under actinic light was significantly higher in roots of the Osgs1;1 mutant than in roots of the Osgs1;2 mutants and the corresponding controls (Fig. 7). The levels of ΦPI and photochemical quenching (PQ) of chlorophyll fluorescence in roots of the Osgs1;1 mutant were higher than those in roots of the controls during illumination, whereas the quantum yield of nonphotochemical quenching [Φ(NPQ)] was lower in the Osgs1;1-mutant roots than in the roots of the other genotypes. These observations suggest that, in Osgs1;1-mutant roots, PSII is active with respect to photochemical reactions and that the reducing power produced by PSII is transported, via the electron transport chain, for use in photosynthetic dark reactions. When wild-type roots were grown in hydroponic culture conditions under no light, no maximum photochemical efficiency of PSII photochemistry (Fv/Fm) was observed in the dark-adapted state (Supplemental Fig. S22). These results suggest that detected photosynthetic parameters in Osgs1;1 mutant roots were a specific response to exposure of root parts to weak light.

DISCUSSION

Osgs1 Mutant Roots Are Useful for the Investigation of Hetero- and Autotrophic Growth

In this study, we first aimed to dissect the pivotal functions of OsGS1;1 and OsGS1;2 in ammonium assimilation and organic N assimilation, which contribute to biomass production in rice. We used ammonium as the N source because assimilation of ammonium is the initial step in the production of Gln (as a form of organic N) in roots via the enzymatic reaction of OsGS1. Rice can grow well in the presence of an adequate concentration of ammonium (several millimolars) as the sole N source, whereas other plants generally exhibit growth reduction at high concentrations of ammonium (Britto et al., 2001a, 2001b; Li et al., 2014; Esteban et al., 2016). Thus, in this way, an understanding of N use by rice plants can simplify the design of experiments aimed at clarifying relationships between ammonium assimilation and central metabolism.

The shoot and root growth of wild-type seedlings was observed to determine when the growth of rice seedlings relied on heterotrophic growth, with nutritional resources supplied by the seed (Bewley, 2001). Growth of the wild-type seedlings was stimulated after the fourth-leaf stage, whereas seed content was estimated to be almost zero by the fourth-leaf stage (Supplemental Fig. S6). By contrast, shoot and root
Figure 7. Chloroplast development and PAM chlorophyll fluorescence analysis in OsGSI;1 mutant roots. A, Cross sections of OsGSI;1 mutant, OsGSI;2 mutant, and wild-type (WT) roots 2 cm from the basal plate (MZ V) were analyzed by TEM. Black arrows identify chloroplasts or plastid. Each region surrounded by a dashed rectangle (i–iii) was expanded. Chloroplasts were observed in the cortex (i and ii), but not in the Endo/Peri/Stele region (iii). We analyzed three biological replicates of each sample. Two independent OsGSI;1 mutants (NC2373, NC; ND8037, ND) were used for TEM analysis. Scale bars = 0.5, 1.0, and 20.0 μm. B, Chlorophyll fluorescence parameters $\Phi_0$, $\Phi(NPQ)$, and $qP$ in OsGSI;1 mutant and OsGSI;2 mutant rice roots, and the corresponding controls were analyzed by the PAM technique. Actinic light treatment (100 μmol photons m$^{-2}$ s$^{-1}$) was started after a 30-s dark relaxation period. Number of biological replicates: $n = 4$. Error bars represent SD. We used mutant NC2373 for PAM analysis. Epi, epidermis; Exo, exodermis; Scl, sclerenchyma; Endo, endodermis; Per, pericycle.

Metabolomic and Transcriptomic Changes Associated with the Two Isoenzymes of OsGSI at the Third-Leaf Stage

The metabolite profiles in OsGSI;1 mutant roots clearly revealed abnormal accumulation patterns of organic C and N metabolites involved in central metabolism (Figs. 2 and 3; Supplemental Datasets S3 and S4). The results of GAM-based network analysis of OsGSI;1 extracted active metabolic subnetworks in Suc metabolism (Fig. 6). Comprehensive GAM analysis allows a systems-wide comparison of biological states, though preassembled metabolic reaction networks should be prepared for each species (Hiemer et al., 2019). Unlike OsGSI;1 knockout plants, OsGSI;1 overexpression lines exhibited no marked change in root biomass, ammonium uptake at the tillering stage, or grain yield (Tabuchi et al., 2005; Bao et al., 2014). These findings suggest that an appropriate level of OsGSI;1 expression is necessary to maintain healthy growth in rice (Figs. 2, 3, and 6). In our previous study, Gln and Asn levels were decreased in the root profiles of OsGSI;1 mutants relative to the wild type (Kusano et al., 2011b), whereas these parameters were increased in this study (Fig. 2). One of the possible explanations for this discrepancy is the metabolic balance between Gln and Asn levels, as indicated by significant positive correlation analysis between the levels of the two amino acids.
(Supplemental Table S2). As Gln and Asn showed close coordination in the absence of a functional OsGS1;1 in the OsGS1;1 mutant, amino acid levels were likely to be decreased or increased in response to Gln and Asn status (Figs. 2 and 6).

Our untargeted metabolite profiling also revealed that OsGS1;2 is mainly involved in maintenance of amino acid levels, whereas it does not influence C metabolism in roots (Figs. 2 and 3). This result underlines that OsGS1;2 mainly functions in the assimilation of ammonium to Gln and other amino acids at the root surface (Ishiyama et al., 1998; Tabuchi et al., 2007; Funayama et al., 2013).

When the transcript abundance of G52 was strongly decreased or disrupted, the expression of GS1-encoding gene(s) was increased in Arabidopsis and Lotus japonicus (García-Calderón et al., 2012; Pérez-Delgado et al., 2013; Betti et al., 2014; Osanai et al., 2017). However, the transcript abundance of OsG52 did not change in the OsGS1;1 mutants (Kusano et al., 2011b). This finding suggests that GS2 cannot compensate for loss-of-function of GS1;1 in rice roots.

OsGS1;1 Causes Changes in Transcript Abundances of Photosynthesis-Related Genes, Including OsSIGs but Not OsGLKs, to Achieve Functional Chloroplast Development at the Third-Leaf Stage in Root Cells

Transcript profiles in roots of the Ogss1;1 mutant, but not those of the Ogss1;2 mutant, included significant increases in the expression of genes associated with light harvesting, light reactions, other aspects of photosynthesis, the Calvin-Benson cycle, and tetrapyrrole biosynthesis (Fig. 4; Supplemental Figs. S10–S12). These results supported the observation of chloroplast development in roots of the OsGS1;1 mutant. In general, chloroplast development is not observed in roots when plants are grown under light-impaired conditions. However, plant roots can develop functional chloroplasts when grown in growth medium with Suc under continuous-light conditions (Nakamura et al., 2009; Kobayashi et al., 2012, 2013), in response to shoot detachment and continuous light exposure (Kobayashi et al., 2012, 2017), and in roots treated long-term with continuous light (Oliveira, 1982). Greening roots of Lens culinaris exhibited active Rubisco and photosynthetic machinery (Nato and Deleens, 1975a, 1975b). The rice Rubisco SMALL SUBUNIT1 gene (OsRbcS1) is not expressed in the main photosynthetic tissue (i.e. LB) but is expressed in nonphotosynthetic tissues, including vascular bundles in roots (Morita et al., 2014). Tobacco (Nicotiana tabacum) has a photosynthetic system in cells around the vascular bundles (Hibberd and Quick, 2002). These findings suggest that plants have a potentially functional photosynthetic system in nonphotosynthetic tissues. In this study, we used a hydroponic culture setup in which black mesh was used to support rice seedlings. The mesh reduced light intensity by shading the roots from light. Furthermore, water can absorb light; infrared and ultraviolet light are the wavelengths most attenuated by water, whereas red and green light are the least affected. The roots of rice grown in paddy fields are potentially under low-light exposure because the diameter of soil particles (“mud”) suspended in the water is about 0.06 mm in a paddy field. The hydroponic culture conditions applied in this study might partly mimic the light environment in a paddy field.

Under such reduced-light conditions, no mature chloroplasts were observed in the roots of Ogss1;2 mutants, the Ogss1;2-complemented lines, or the wild type, whereas Ogss1;1 mutant roots exhibited mature chloroplasts. These results suggest that chloroplast development in Ogss1;1 mutant roots may be mediated by up-regulation of OsSIGs (Fig. 5). TEM and PAM chlorophyll fluorescence analyses of the Ogss1;1 mutant roots revealed that their chloroplasts had functional electron transport chains, whereas those of Ogss1;2 and the wild type did not (Fig. 7; Supplemental Fig. S19). These results indicate the importance of OsGS1;1, at least partly, in the expression regulation of photosynthesis-related genes in rice roots.

Chloroplast development is thought to be regulated by phytohormone signaling (Waters et al., 2009; Kobayashi et al., 2012, 2013), plastid-to-nucleus retrograde signaling (Kakizaki et al., 2009; Petrillo et al., 2014), and light-dependent regulators of transcription factor genes, namely GLKs, GNC, and CGA1 (Chiang et al., 2012; Hudson et al., 2013; Behringer et al., 2014; Behringer and Schwechheimer, 2015). Overexpression experiments revealed that GLKs play roles in chloroplast biogenesis and up-regulation of photosynthesis-related genes in monocots and dicots (Rossini et al., 2001; Nakamura et al., 2009; Waters et al., 2009; Powell et al., 2012; Kobayashi et al., 2013). Given that the transcription of AIGLKI, AIGATA, and AICGAI genes in Arabidopsis is induced by nitrate and/or organic N treatments (Wang et al., 2003; Price et al., 2004; Scheible et al., 2004; Bi et al., 2005; Gutiérrez et al., 2008), chloroplast development and N metabolism are likely to be tightly coordinated in plants. In this study, we did not observe significant changes in OsGLK transcript abundance in Ogss1;1 mutant roots relative to the control (Fig. 5). The OsGNC transcript abundance was slightly elevated, whereas the OsGATA1 transcript abundance was below the limit of detection of our microarray data (Supplemental Table S7). This suggests that chloroplast development in roots of the Ogss1;1 mutant might have occurred via other mechanisms, although additional studies would be required to support this hypothesis. A possible mechanism for the chloroplast development we observed in Ogss1;1-mutant roots is activity contributed by the OsSIG genes (Fig. 5). SIGs are crucial for tuning nuclear-encoded RNA polymerase, which is involved in chloroplast biogenesis and maintenance (Kasai et al., 2004; Kubota et al., 2007; Lysenko, 2007; Tozawa et al., 2007; Belbin et al., 2017).
CONCLUSION

GS is one of the most exhaustively studied enzymes in living organisms (Pesole et al., 1991). In this study, we demonstrate the importance of GS1;1 in rice by investigating the relationships between specific phenotypes, including metabolomic and transcriptomic changes; performing microscopic tissue studies; and conducting two types of network analyses, namely gene correlation network analysis and integrated GAM analysis. These results highlight the distinct roles and impacts of OsGS1;1 and OsGS1;2 in plant growth, central metabolism, and plastid development. To obtain deeper insights into hidden C- and N-regulatory networks and chloroplast biogenesis mediated by OsGS1;1, future work must focus on the identification of the key factor(s) involved in regulation of OsGS1;1 transcription, epigenetics, and translation (Patil and Nielsen, 2005; Ushijima et al., 2017). Such investigations will illuminate global and spatial views of regulatory mechanisms triggered by GS1 in central metabolism.

MATERIALS AND METHODS

Plant Materials

Oryza sativa ssp. japonica (‘Nipponbare’) was the wild-type rice material chosen for this study. We obtained seeds of homozygous OsGS1;1 specifically, two independent mutants, NC2373 and ND9037, OsGS1;2 knockout mutants, and the OsGS1;2-complemented lines as reported elsewhere (Tabuchi et al., 2005; Funayama et al., 2013). Null plants segregated from heterozygous OsGS1;1 and wild-type plants, respectively, were used as controls for these mutants. Plants were grown under hydroponic culture conditions as previously described (Kusano et al., 2011b) with modifications. Detailed information is provided in the Supplemental Materials and Methods.

Changes in wild-type seed weight at the growth periods from the second- to sixth-leaf stage were determined as follows. Wild-type seedlings were grown under hydroponic culture conditions. The remaining seed tissue was taken from each seedling at each growth stage. These seeds (12 sixth-leaf stage) were determined as follows. Wild-type seedlings were grown in the Supplemental Materials and Methods. Plants and the Supplemental Materials and Methods.

Detection of Promoter Elements

We searched for the promoter elements identified by rice local distribution of short sequence analysis (Yamamoto et al., 2007; Hieno et al., 2014), which include the TATA box, Y patch, GA element, CA element, and regulatory element group, in OsGS1;1 and OsGS1;2 promoter sequences from −1000 to −1 bp relative to the top transcription start site (TSS) of the genes. The search revealed a perfect match between the reported octamer sequences corresponding to the promoter element groups. The top TSSs were identified using large-scale TSS-sequencing analysis (K. Kusunoki, M. Tokizawa, T. Ushijima, T. Matsushita, Y. Kanesaki, Y. Suzuki, H. Koyama, and Y.Y. Yamamoto, unpublished data). Determination of sequence similarity between the two promoter sequences was conducted using BLAST.

Gene Coexpression and Network Inferences in Shoots and Roots

To conduct condition-dependent coexpression networks, we collected 17 independent datasets (203 microarrays in total) sampled from shoot tissues in rice (Supplemental Table S1). For roots, we used 22 independent datasets (203 microarrays in total; Supplemental Table S2). All CEL files for publicly available datasets based on the Affymetrix GeneChip Rice Genome Array were downloaded from the NCBI GEO (Barrett et al., 2013). We preprocessed/summarized the raw CEL files within each dataset by the robust multichip average (Irizarry et al., 2003) with the ‘affy’ package (Gautier et al., 2004) in the R program v2.14.1 (http://www.R-project.org). We also normalized the resulting log2 values by means-based scale normalization (Fukushima et al., 2009). We eliminated any probe set at risk of cross hybridization (‘x_at’ and ‘s_at’) and probe sets with the prefix ‘RPTR’ or ‘AFFX.’ To interrogate gene regulatory networks/metabolic pathways involved with OsGS1 genes, we focused on transcription factor genes from Caldana et al. (2007), metabolic enzyme genes from RiceCyc version 3.3 (Youens-Clark et al., 2011) and MapMan bins (Usadel et al., 2005), phytohormone biosynthesis genes, and genes involved in nutrient transport, resulting in expression data matrix consisting of 16,041 probe sets for roots and 15,965 probe sets for seedlings, respectively. We measured gene coexpressions by both the Pearson and Spearman correlation coefficients. Furthermore, we used three information-theoretic inference methods, CLR (Faith et al., 2007), ARACNE (Margolin et al., 2006), and MRNET (Meyer et al., 2007), to infer regulatory relationship between genes. These inferences were based on the ‘minet’ package (Meyer et al., 2007). We used the annotation of each gene in the latest CSV file Rice.na32.annot.csv (June 2011) released by Affymetrix for the GeneChip Rice Genome Array.

GO Analysis

To retrieve the relevant annotations by statistically assessing the enrichment of GO terms in clusters, we used BINGO (Maere et al., 2005) for the analysis of significantly overrepresented GO categories among genes coordinated with OsGS1. The hypergeometric test for each functional category was adjusted by the Benjamini and Hochberg FDR for multiple testing (Benjamini and Hochberg, 1995).

Metabolite Profiling

Metabolite profiling was performed as described elsewhere (Kusano et al., 2011b) with modifications. For details, see the Supplemental Materials and Methods. We used six biological replicates for each of the OsGS1;1, OsGS1;2, and wild-type samples; as controls, null samples were used (n = 12).

Quantification of Starch Content

Frozen root powder (20 mg fresh weight) per sample was used for the analysis (number of biological replicates, n = 3). Starch quantification was conducted as described in the Supplemental Materials and Methods.

RNA Isolation

Approximately 20 to 40 mg fresh weight of root tissue harvested from each biological replicate was crushed (1 min, 4°C) with 5-mm zirconia beads in a TissueLyser (Qiagen RNase-free DNase set). The extracted RNA was quantified using the RNAeasy plant mini kit (Qiagen) with on-column DNase treatment (Qiagen RNase-free DNase set). The extracted RNA was quantified on a NanoDrop ND-1000 spectrophotometer. For each sample, 1 μL of RNA (83.3 ng μL−1 dissolved in RNase-free water) was subjected to quality assessment on a BioAnalyzer 2100 and the Series II RNA 6000 nano kit (Agilent Technologies). Two or three biological replicates were used for each sample for RNA isolation.

Transcript Profiling

mRNA transcript abundance was analyzed using rice Affymetrix GeneChip microarrays and customized oligo microarrays containing 56,894 rice genes (Agilent Technologies).

For Affymetrix microarray analysis, labeled complementary DNA (cDNA) synthesis, array hybridization, and normalization of the signal intensities were performed according to instructions provided by the manufacturer. In brief, biotinylated cRNA was labeled with a biotinylated nucleotide analog/ribonucleotide mix using the GeneChip 3’ IVT express kit (Affymetrix). After fragmentation, 12.5 μg of cRNA was hybridized for 16 h on GeneChip rice genome arrays (Affymetrix #900129). After fragmentation, 12.5 μg of cRNA was hybridized for 16 h on GeneChip rice genome arrays (Affymetrix #900129). The GeneChip was washed and stained on an Affymetrix Fluidics station 450 and scanned on a GeneChip scanner 3000 integrated with the Affymetrix microarray suite software. Data were deposited in the NCBI GEO database under accession number GSE49798.

Oligo microarray construction was performed by Agilent Technologies.

Microarray analysis was conducted as described (Maruyama et al., 2014;
RT-qPCR Analysis

The Invitrogen SuperScript III first-strand synthesis system for RT-qPCR was used for first-strand cDNA synthesis. Reverse transcription was performed with 1 μg of total RNA using oligo(dT) 15 as a primer. SYBR Green fluorescence was detected using FastSYBR Green master mix (Thermo Scientific) on an Applied Biosystems StepOnePlus real-time system (Life Technologies). mRNA content was quantified, using a purified cDNA clone as a standard. The sequences of all oligonucleotide primers employed in this study are listed in Supplemental Table S5. Each mRNA value was normalized against the corresponding amount of rice actin 1 mRNA (INSD accession, AK100267). Relative mRNA amounts were log-transformed for statistical data analysis. Significance of differences in relative mRNA content between mutants and the corresponding controls (the null line was the control for Osgs1;1 mutant, and wild-type was the control for Osgs1;2 mutant) was determined using Welch's t test (P < 0.05). Two or three independent biological replicates were used to carry out statistical analyses and to determine summary statistics.

GSEA and MSEA

GSEA of differentially expressed genes (DEGs) was carried out with g:Profiler (https://biit.cs.ut.ee/gprofiler/gost; Raudvere et al., 2019), using the default parameters. MSEA was performed using MetaMophList (https://www.metaboanalyst.ca/MetaMophList/faces/home.xhtml; Xia et al., 2015), using the default parameters. A significant change was set at P < 0.05 for the FDR-adjusted P value.

Integrated Metabolomic and Transcriptomic Network Analysis

To identify and investigate the metabolic and transcriptional subnetworks that differed to the greatest extent between Osgs1;1 mutant and wild-type roots at the third-leaf stage, we performed integrated metabolomic and transcriptomic network analysis using GAM (Sergushichev et al., 2016). The Shiny-based web application (https://artymovlab.wustl.edu/shiny/gam/) can identify the most regulated subpathway based on user-identified DEGs and significantly altered metabolites, based on the KEGG (Kanehisa and Goto, 2000) and MetaboCards (Wishart et al., 2007) databases, respectively. Because the current version of GAM supports metabolic pathway information for only Arabidopsis, we first converted all rice gene IDs in our microarray data (Affymetrix) into Arabidopsis homolog IDs using the BioMart (Durincik et al., 2005) and annotationTools packages (Kuhn et al., 2008) and the The Rice Annotation Project Database (RAP-DB) annotation (Sakai et al., 2013). To retrieve the orthologs, we used HomoloGene v. 68 (http://www.ncbi.nlm.nih.gov/HomoloGene). The same cutoff values were applied for extraction of significant metabolite changes (Fig. 2) and DEGs (Fig. 4). We then integrated 27 metabolites with Human Metabolome Database-identifier (Wishart et al., 2007) and 34 DEGs in the metabolic map with default parameters in GAM. The network consisting of metabolite profiles and microarray data were visualized using Cytoscape 3.5.1. Then, manual curation was conducted on the pathways in each module by comparing pathways in KEGG and OrayaExpress (Hamada et al., 2011).

Ultrasound Analysis of Rice Root by TEM

Cross sections of primary root regions 2.0 cm (MZ V) or 8.0 cm (MZ III) from the basal plate were used for ultrasound analysis. Root sections of the Osgs1;1 mutant, the Ogs1;2 mutant, and wild-type were analyzed by TEM by the method of Toyooka et al. (2000) with minor modifications. Harvested root tissues were immediately fixed for 3 h at room temperature with 4% (w/v) paraformaldehyde, 2% (w/v) glutaraldehyde, and 150 mM sucrose in 100 mM sodium cacodylate buffer (pH 7.4) and post-fixed for 3 h with 1% osmium tetroxide in 50 mM cacodylate buffer (pH 7.4). After dehydration in a graded methanol series, the samples were embedded in Epon812 resin. Images were viewed and recorded on a JEM-1400 (JEOL). Three biological replicates were used for each analysis.

Chlorophyll Fluorescence Analysis

Chlorophyll fluorescence was measured on a Dual-PAM-100 system equipped with a leaf clip holder (Heinz Walz). Roots were washed and rinsed three times in Milli-Q water just before analysis. Then, each root sample at 2 to 3 cm from the basal plate was placed in the PAM instrument. After 15 min incubation in the dark, the minimum chlorophyll fluorescence (F9) was measured under dark conditions, and then a saturating flash of light was applied to determine maximal chlorophyll fluorescence (Fm). After a 30-s dark relaxation period, actinic light treatment (100 μmol photons m−2 s−1) was delivered for 150 s to obtain the fluorescence parameters, steady-state fluorescence (F), and maximal fluorescence in the light (Fm'). After switching off the actinic light, the samples were exposed for 100 ms to far-red light to measure minimal fluorescence (Fm’). Four biological replicates of each sample were used for this analysis. The other photosynthetic parameters (Fv/Fm, Fv’/Fm’, qL, and qP) were calculated using the following equations:

\[ F_v/F_m = (F_m - F_o)/F_m; \]
\[ F_v' / F_m = (F_m - F_o')/F_m; \]
\[ \Phi_L = (F_m - F_o)/F_m; \] and
\[ q_P = (F_m - F_o)/F_m. \]

The quantum yield of light noninduced nonphotochemical quenching (ΦNO) is 1/[NPQ + 1 + qL(Fm/Fo - 1)], where NPQ is (Fm/Fo - Fm’), qL = (Pm/F), and the ΦNPQ is 1 - ΦL - ΦNO (Kramer et al., 2004). Four biological replicates were used for each analysis.

A mini PAM system was used to measure Fv/Fm of wild-type roots with or without exposure of roots to light (Heinz Walz). Ten biological replicates were used for the analysis.

Statistical Data Analysis

Statistical tests were performed using the R statistical software (64-bit, version 3.2.2, http://www.r-project.org/). For each mutant and wild type, log2 values in metabolite content or transcript abundance were calculated in the corresponding profiles. FDR-controlled P values were calculated using the “limma” package in R (Benjamini and Hochberg, 1995) or GeneSpring GX. Microsoft Excel 2013 or 2016 was used for Welch’s t test, and SIMCA version 13.0.0.0 (Umetrics) was used for multivariate analysis (Supplemental Fig. S3).

Accession Numbers

Sequence data from this article can be found in the RAP-DB/GeneBank data libraries under accession numbers Os03g0718100 (ACTIN1), Os02g0735200 (OsGS1;1), Os03g0223400 (OsGS1;2), Os12g0189400 (OsCPD1), Os10g0502400 (OsSIG6), Os04g0234600 (OsGNC), Os09g0346500 (OsLhcb1), Os03g0229100 (OsGLK2), Os01g0502480 (OsHEM), Os12g01799400 (OsPN), Os10g0502480 (OsHEM-A1), Os01g0229100 (OsCPD1), Os04g0234600 (OsBPase), Os09g034600 (OsLhcb1), Os03g0502950 (OsLhcb2), Os08g0163400 (OsSIG1), Os03g0271100 (OsSIG28), Os05g0589200 (OsSIG3), Os08g0242800 (OsSIG6), Os06g0348800 (OsGLK1), AF318582 (OsGLK2), Os06g0571800 (OsGNC), and Os02g020400 (OsCGA1).

Supplemental Data

The following supplemental materials are available.

Supplemental Figure S1. Multiple alignment of GS1 in rice, Arabidopsis, soybean, and maize.

Supplemental Figure S2. Phylogenetic tree of GS1.

Supplemental Figure S3. Comparison of local distribution of octamer sequences in the promoter site of OsGS1;1 and OsGS1;2.

Supplemental Figure S4. Network inference of genes associated with each cytosolic OsGS1.

Supplemental Figure S5. Shoot and root growth of two independent OsGS1;1 mutants and wild-type plants at the fourth-leaf stage.
Supplemental Figure S6. Dry weight of total (sum of shoot, root, and seeds), shoot, root, and seed biomass.

Supplemental Figure S7. Scatter plots of PCA of the metabolite profiles of Osgs1 mutant samples and their controls.

Supplemental Figure S8. Changes in the metabolite profiles of Osgs1;1 mutant and Osgs1;2 mutant at the third-leaf stage.

Supplemental Figure S9. Venn diagram comparing differentially expressed genes in roots of Osgs1;1 and Osgs1;2 mutants.

Supplemental Figure S10. Metabolism overview of transcript changes in probe sets in the MapMan tool for root samples of two independent knockout mutants of Osgs1;1 and one mutant for Osgs1;2 compared to their respective controls.

Supplemental Figure S11. Overview of transcript abundance changes of genes of the Calvin-Benson cycle in probe sets in the MapMan tool for root samples of two independent mutants of Osgs1;1 and one mutant of Osgs1;2 compared to their respective controls.

Supplemental Figure S12. Overview of transcript changes of tetrapyrrole biosynthesis in probe sets in the MapMan tool for wild-type at the third- and fourth-leaf stages.

Supplemental Figure S13. Overview of transcript abundance changes in probe sets in the MapMan tool for wild-type at the third- and fourth-leaf stages.

Supplemental Figure S14. mRNA content of photosynthesis-related genes in the profiles of Osgs1;1 versus wild-type, quantified by RT-qPCR at the third- and fourth-leaf stages.

Supplemental Figure S15. Overview of GSEA of the upregulated transcript profiles in the Osgs1;1 mutant compared to wild type at the third-leaf stage.

Supplemental Figure S16. Overview of GSEA of the downregulated transcript profiles in the Osgs1;1 mutant compared to wild-type at the third-leaf stage.

Supplemental Figure S17. Overview of MSEA of the increased metabolite profiles in the Osgs1;1 mutant compared to wild type at the third-leaf stage.

Supplemental Figure S18. Cross section of root cortex regions of two independent mutants of Osgs1;1 and the wild type as observed by TEM of two MZs.

Supplemental Figure S19. TEM analysis of cross sections of Osgs1;2 mutant and wild-type roots.

Supplemental Figure S20. TEM analysis of cross sections of Osgs1;2-complemented line and wild-type roots.

Supplemental Figure S21. PAM chlorophyll fluorescence analysis of Osgs1;1 mutant (NC2373 line) and Osgs1;2 mutant roots and the corresponding controls.

Supplemental Figure S22. Fv/Fm values of wild-type roots grown under low-light conditions (dark) or in the presence of light (light).

Supplemental Table S1. Effect on shoot and root growth in the two independent mutants of Osgs1;1 (NC2373 and ND8037) and the one Osgs1;2 mutant compared to the corresponding control plants.

Supplemental Table S2. Correlation coefficient between Gln and Asn levels in the two independent mutants of Osgs1;1 (NC2373 and ND8037), the Osgs1;2 mutant, and the corresponding controls.

Supplemental Table S3. Effect of 3% exogenous Suc addition on shoot and root growth of wild-type plants.

Supplemental Table S4. Effect of 3% exogenous Suc addition on transcript abundances of photosynthesis-related genes in wild-type plants.

Supplemental Table S5. Up- and down-regulated genes in the Osgs1;1 (NC2073) mutant versus wild type at the third-leaf stage (A) and the fourth-leaf stage (B).

Supplemental Table S6. Significantly changed genes in Osgs1;1 mutants versus wild type categorized into molecular function classes.

Supplemental Table S7. Transcript abundance changes and normalized signal intensities of OsGNC and OsCCAA1 in Osgs1;1 mutant roots versus wild type.

Supplemental Table S8. Abbreviations of gene names, KEGG IDs, and KEGG pathway names of each corresponding gene in Figure 6.

Supplemental Table S9. Abbreviations of metabolite names in Figure 6.

Supplemental Table S10. Maximum quantum efficiency of PSII in root samples of the Osgs1;1 and Osgs1;2 mutant and the corresponding controls.

Supplemental Dataset S1. Gene network inference of genes associated with OsGS1;1 and OsGS1;2 for public microarray data of rice shoots.

Supplemental Dataset S2. Gene network inference of genes associated with OsGS1;1 and OsGS1;2 for public microarray data of rice roots.

Supplemental Dataset S3. The log2 FCs of 82 metabolite levels in Osgs1;1 and Osgs1;2 mutants compared to the corresponding controls at the third-leaf stage.

Supplemental Dataset S4. The log2 FCs of 85 metabolite levels in roots of the Osgs1;2-complemented lines compared to the corresponding control at the third-leaf stage.

Supplemental Dataset S5. Photosynthesis-related genes that were significantly upregulated in the two independent Osgs1;1 mutants, NC2373 and ND8037.

Supplemental Dataset S6. Primer sequences used for (RT-q)PCR assays for validation of transcript abundance changes in Affymetrix and Agilent microarray data.

Supplemental Materials and Methods. Metabolomics metadata.

ACKNOWLEDGMENTS

The authors thank Mikiko Koizumi, Dr. Fumiyoshi Myouga, Koji Takano, Sachiko Ooyama (RIKEN), and Takayuki Fujita (Tohoku University) for technical assistance; and Bertrand Hirel (Institute National De La Recherche Agronomique, France), Mitsue Miyao, Dr. Toshihiko Hayakawa, Dr. Keiki Ishiyama, Dr. Miwa Ohashi (Tohoku University), and Hiroshi Shiba (Gene Research Center, Tsukuba Plant Innovation Research Center, University of Tsukuba) for their advice. We are grateful to Dr. Per Jonsson and Dr. Hans Stenhund of Umeå University (Sweden), and Thomas Moritz of the Swedish Metabolomics Centre for providing the customized software for pretreatment of gas chromatography-mass spectrometry data.

Received September 12, 2019; accepted January 9, 2020; published February 5, 2020.

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Role of OsGS1;1 in Metabolic Balance in Roots

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