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Cytosolic glutamine synthetase1;2 is responsible for the primary assimilation of ammonium in rice roots

Kazuhiro Funayama[§], Soichi Kojima[§], Mayumi Tabuchi-Kobayashi, Yuki Sawa, Yosuke Nakayama, Toshihiko Hayakawa and Tomoyuki Yamaya[*]
Graduate School of Agricultural Science, Tohoku University, Sendai, 981-8555, Japan

Among three genes for cytosolic glutamine synthetase (*OsGS1;1*, *OsGS1;2*, and *OsGS1;3*) in rice (*Oryza sativa* L.) plants, the *OsGS1;2* gene is known to mainly be expressed in surface cells of roots, but its function was not clearly understood. We characterized knockout mutants caused by the insertion of an endogenous retrotransposon *Tos17* into exon-2 of *OsGS1;2*. Homozygously inserted mutants showed severe reduction in active tiller number and hence panicle number at harvest. Other yield components, such as spikelet number per panicles, 1,000-spikelet weight, and proportion of well ripened grains, were nearly identical between the mutants and wild-type plants. When contents of free amino acids in roots were compared between the mutants and wild type, there were marked reduction in contents of glutamine, glutamate, asparagine, and aspartate, but a remarkable increase in free ammonium ions in the mutants. Concentrations of amino acids and ammonium ions in xylem sap behaved in a similar fashion. Re-introduction of *OsGS1;2* cDNA under the control of its own promoter into the knockout mutants successfully restored yield components to wild-type levels as well as ammonium concentration in xylem sap. The results indicate that GS1;2 is important in the primary assimilation of ammonium ions taken up by rice roots, with GS1;1 in the roots unable to compensate for GS1;2 functions.

Introduction

Nitrogen availability is the limiting factor for plant growth and productivity in most environments. Under anaerobic conditions in paddy fields where rice plants are cultivated, NH_4^+ is a major form of available inorganic nitrogen source. This is in contrast to other plants grown in aerobic field conditions, where NO_3^- is predominant (Yamaya and Oaks, 2004). On the other hand, excess NH_4^+ is toxic to most plants (Kronzucker et al., 2001; Hachiya et al., 2012; Li et al., 2012). Therefore, NH_4^+ transport and subsequent assimilation systems should be efficiently regulated in rice roots. Real-time monitoring of NH_4^+ transport by positron emitting tracer imaging systems showed that the translocation of ^{13}N, taken up by roots, to top parts was completely inhibited by methionine sulfoximine, an inhibitor of glutamine synthetase (GS, EC 6.3.1.2) (Kiyomiya et al. 2001). The major forms of nitrogen in xylem sap of rice plants are glutamine and asparagine (Fukumorita and Chino, 1982) and asparagine is synthesized from glutamine (Lea et al. 2007). Taken together, these results strongly suggest that most of the NH_4^+ taken up by the roots is assimilated within the roots by GS. GS catalyzes an ATP-dependent conversion of glutamate to glutamine using NH_4^+ (Ireland and Lea, 1999). Since the discovery of GS/glutamate synthase (GOGAT) cycle by Lea and Miflin (1974), it is now well established that this cycle is the only route for the primary assimilation of NH_4^+ in plants grown under normal conditions (Ireland and Lea, 1999). In addition to soil and fertilizer derived NH_4^+, plants generate NH_4^+ in the course of several metabolic processes, such as photorespiration, nitrate reduction, phenylpropanoid biosynthesis, and catabolic processes during senescence (Ireland and Lea, 1999; Yamaya and Oaks, 2004). A small gene family has been identified that encodes GS1 in various plants and recent phylogenic analysis for GS1 in Poaceae species showed three distinct groups corresponding to "GS1", "GSr", and "GSe" (Swarbreck et a., 2011). According to the phylogenic analysis, "GS1" and "GSe" groups are more related to each other than to the "GSr" group. In rice, OsGS1;1, OsGS1;2, and OsGS1;3 were identified (Tabuchi et al., 2007), as in groups of "GS1", "GSr", and "GSe", respectively (Swarbreck et a., 2011). However, detectable mRNA for *GS1;1* in roots, that for *GS1;2* in leaf blade, and mRNAs for both *GS1;1* and *GS1;2* in

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Correspondence
*Corresponding author,
e-mail:tyamaya@biochem.tohoku.ac.jp

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spikelet at the early stage of ripening was also seen in rice, respectively (Tabuchi et al., 2007). This seems also true in barley that recent study by Goodall et al. (2013) showed the distinct function of three GS isoenzymes, such as HvGS1_1, HvGS1_2, and HvGS1_3. Our previous study with knockout mutants clearly showed that GS1;1 is important in the processes of nitrogen remobilization though the phloem from source organs and grain filling (Tabuchi et al., 2005). High concentration of glutamine and asparagine, as the major forms of remobilized nitrogen, were detected in rice phloem sap (Hayashi and Chino 1990) and the slightly alkaline solution of the phloem sap (Nishiyama et al., 2012) probably makes it possible to solubilize these amides as high as 20-30 mM. Strong evidence was also obtained that GS1-3 and GS1-4, both in "GS1" group, in maize are important in grain filling (Martin et al., 2006). Another recent study using knockout mutants demonstrated that GLN1;2, the most strongly expressed GS1 genes in leaves, is important in vegetative biomass production in *Arabidopsis thaliana* (Lothier et al., 2011). In contrast to the "GS1" in several plants, no clear evidence for the functions of "GSr", such as GS1;2 in rice and Gln1-1 and Gln1-5 in maize, has been obtained. Two NADH-GOGAT genes have been identified in rice. These are *OsNADH-GOGAT1* and *OsNADH-GOGAT2*. As there are multiple tissue and age dependent routes for NH_4^+ incorporation in plants, one form of GS1 and NADH-GOGAT is responsible for the majority of NH_4^+-assimilation in the plants, with another form responsible for the assimilation of NH_4^+ produced during photorespiration. It is well known from work in barley mutants that GS2/Fd-GOGAT are responsible for the assimilation of photorespiratory released NH_4^+ (Wallsgrove et al., 1987; Kendall et al., 1986). However, these barley mutants lacking either GS2 or Fd-GOGAT are able to grow normally under non-photorespiratory conditions. This suggests that GS1 and NADH-GOGAT are apparently important in the normal development and growth of plants (Yamaya and Oaks, 2004).

As in the photorespiration mutants, reverse genetic approaches are powerful for elucidating gene function. In addition to the mutants lacking GS1;1 as already described (Tabuchi et al., 2005) we isolated mutants for two NADH-GOGAT species (Tamura et al., 2010, Tamura et al., 2011) in rice using retrotransposon *Tos17* insertion mutants (Hirochika et al., 1996). Rice seeds of various lines mutated by the random insertion of an endogenous retrotransposon, *Tos17*, into the rice genome are now available from the Mutant Panel, Rice Genome Resource Center (http://www.rgrc.dna.affrc.go.jp/index.html), National Institute of Agrobiological Sciences, Japan, upon request. The results show NADH-GOGAT1 is important in the development of active tiller number and hence panicle number of rice (Tamura et al., 2010). NADH-GOGAT2 is apparently important in controlling spikelet number per panicle (Tamura et al., 2011). None of the corresponding isoenzymes is able to compensate for the functions of GS1 or NADH-GOGAT. Our previous works on the profile of tissue and cell-specific expression of *OsGS1;2* showed a rapid, but transient increase in transcript abundance in rice seedling roots following NH_4^+ supply, although lower expression of this gene was observed in other organs (Tabuchi et al., 2007). Recent transcriptome analysis also suggested higher *OsGS1;2* expression in root (Hamada et al., 2011). The expression of *OsGS1;2* was detected in the dermatogen, epidermis, and exodermis within 3 to 6 hr following NH_4^+-supply (Ishiyama et al., 2004a). The kinetic properties of GS1;2 purified from recombinant protein overexpressed in Escherichia coli showed that this enzyme was a high-affinity subtype for NH_4^+ with relatively high Vmax values (Ishiyama et al., 2004a), as compared with the major high-affinity GS1 in Arabidopsis thaliana (Ishiyama et al., 2004b). This suggests that GS1;2 is apparently important in the primary assimilation of NH_4^+ by rice roots. Recent study also suggested that GS1_2 was the major form in the roots and might be responsible for NH4+ assimilation in roots of barley (Goodall et al., 2013). However, conclusive evidence has not been obtained to support our hypothesis.

The current paper describes the characteristics of *OsGS1;2*-knockout mutants that were successfully isolated from the mutant pool. The possible function of GS1;2 and genetic evidence for its fundamental role in productivity of rice plants is discussed.

Materials and Methods

Plant Materials

The Project for Rice Genome Research, the Ministry of Agriculture, Forestry and Fisheries of Japan (mutant panel: http://tos.nias.affrc.go.jp/~miyao/pub/tos17/) kindly provided 20 seeds each from seven lines (NF6484, NF6568, NF6584, NF6472, NF6488, NF6572, and NF6588) of rice (*Oryza sativa* L. cv Nipponbare) in which the retrotransposon *Tos17* could potentially have been inserted into the *OsGS1;2* gene. For the isolation of knockout mutants, the germinated seeds of those candidate lines were transplanted on a synthetic culture soil (Obara et al., 2001) in a plastic tray (14 x 32 cells, 15 mm in diameter x 30 mm height) and grown for 15 days in a

greenhouse controlled at 26 °C with supplemental light from 5:30 am to 6:30 pm. Genomic DNA was extracted from 2 g fresh weight of fully expanded leaf blade at the second position from the bottom. Leaves were harvested, immediately frozen in liquid nitrogen and stored at -80 °C for DNA sequencing, as well as the position of *Tos17* insertion into the *OsGS1;2* gene determined by PCR and sequencing as described below. Line NF6488 was successfully identified as the insertion mutant for GS1;2. NF6488 plants were self-fertilized and a segregated homozygote for Tos17 was used as a OsGS1;2 knockout mutant. For the quantitative real-time PCR analysis and assay for contents of amino acids, total nitrogen and assay of GS activity, the knockout mutants were grown hydroponically in the presence of 1 mM NH_4Cl for 18 days in an outdoor growth chamber, as described previously (Tamura et al., 2010), and whole roots harvested, weighed, and stored at -80 °C until required. The mutants were also grown hydroponically for 35 days and the shoots were cut off at 3 cm above the stem base. The sap exuded by root pressure for the first 15 min was discarded and the sap in the next 40 min was collected and stored at -80 °C. As a control, wild-type Nipponbare plants were cultivated under the same conditions. When phenotypic characteristics of the mutants were compared with the wild-type Nipponbare, these rice plants were grown in paddy field in Kashimadai, Miyagi, Japan, until the ripening stage in 2009 and 2010, as described previously (Obara et al., 2004, Tamura et al., 2010). A no-insertion null line, in which *Tos17* in *OsGS1;2* gene was lost during the self-fertilization, was used as a control plant in 2009. In 2010, wild type Nipponbare was used as a control plant for the field test.

Screening for the GS1;2 mutant and identification of insertion position

Seven candidate lines (NF6484, NF6568, NF6584, NF6472, NF6488, NF6572, and NF6588) as a Tos17 insertion and two lines (PFG_1B-10606.R and PFG_3D-00345.R) as a T-DNA insertion into *OsGS1;2* gene were tested for isolation of mutants. Preparation of genomic DNA and DNA gel-blot analysis were performed as described previously (Tabuchi et al., 2005). For the isolation of *Tos17*-insertion mutants, two *Tos17*-specific primers (Tabuchi et al., 2005) and two OsGS1;2-specific primers (*GS1;2F180-205*: 5'-*CGTCTGCACTAGCAAGTCTCCTGCTG*-3' and *GS1;2R922-897*: 5'-*CTTTCGTTCCCTTCCTTTTGGCCAT*C-3') were used for PCR amplification of 50 ng of genomic DNA from each pooled DNA sample. To test the T-DNA insertion, both left border- and right border-specific primers for T-DNA and *OsGS1;2*-specific primers recommended by Primer3 (http://frodo.wi.mit.edu/primer3/), a tool for designing primers, were used for PCR. When the insertion position of *Tos17* in *OsGS1;2* gene was determined, the amplified DNA was first purified with MontageTM PCR Centrifugal Filter Devices (Merck Millipore, Billerica, MA, USA) according to the instruction protocol. PCR amplification was carried out using OsGS1;2 forward primer (*GS1;2F180-205*: 5'-*CGTCTGCACTAGCAAGTCTCCTGCTG*-3'), *Tos17* forward primer (5'-*TTGGATCTTGTATCTTGTATATAC*-3'), and OsGS1;2 reverse primer (*GS1;2R922-897*: 5'-*CTTTCGTTCCCTTCCTTTTGGCCATC-3*') and the amplified DNA was sequenced using Big Dye Terminator Cycle Sequencing Ready Reaction Kit version 3.1 (Applied Biosystems, Tokyo, Japan).

RT-PCR and quantitative real-time PCR

Total RNA was extracted from the whole roots of 18-days-old seedlings of the mutants and wild-type Nipponbare and the first strand cDNA was synthesized, as described previously (Tamura et al., 2010). RT-PCR analysis was carried out using *OsGS1;2* mRNA-specific primers as follows: forward primer (*GS1;2cDNAF732-755*: 5'-*GTTGGAGGATCGGGCATAGACCTC*-3'), and reverse primer (*OsGLN1;2-RR* (Ishiyama et al., 2004a), 5'-*CACTTGGTCAGCAGCGGCGATGCCAACT*-3'). The set of primers for detection of actin mRNA was used as follows: forward primer, 5'-*CTTCATAGGAATGGAAGCTGCGGGTA*-3' and reverse primer, 5'-*CGACCACCTTGATCTTCATGCTGCTA*-3' (Sonoda et al., 2003). PCR amplification and electrophoresis were carried out as described previously (Tabuchi et al., 2005). Amplified fragments were all sequenced to confirm their reliability. Quantitative real-time PCR analysis was performed using gene specific primers for *OsGS1;1* (Ishiyama et al., 2004a), *OsGS1;2* (Tabuchi et al., 2005), *OsNADH-GOGAT1* and *OsNADH-GOGAT2* (Tabuchi et al., 2007), *OsGS2* (Tamura et al., 2011), and *OsAMT1;2* and *OsAMT1;3* (Sonoda et al., 2003). Primers for *OsAS* were used as follows: forward primer, 5'-*TCCCAAGAATGCTGCTAGGT-3*' and reverse primer, 5'-*CGACCAGATGGATCAAGGTT-3*'. The PCR products were detected as described previously (Tamura et al, 2010).

Assay of total nitrogen, amino acids and GS activity

Knockout mutant and wild type Nipponbare plants were grown in a paddy field as described below and whole top parts were harvested at 54 days (vegetative stage)

and 111 days (reproductive stage) after transplanting. The samples were air-dried, weighed, powdered with a Multi-Bead Shocker MB601U (Yasui Co. Ltd., Tokyo, Japan), and stored in a desiccator. Total nitrogen content was determined using an Elemental Analyzer, as described previously (Tamura et al., 2011). Four or five replicates roots and the third leaf blades of the knockout mutant and Nipponbare seedlings, grown hydroponically for 18 days were harvested, weighed, and stored at -80 °C until required. Frozen samples were powdered in liquid N2 using a Mixer Mill MM300 (QIAGEN, Hilden, Germany) at 25Hz for two min. Extraction and determination of amino acids and ammonium ions were performed as described previously (Tabuchi et al., 2005). For the GS assay, crude extracts were prepared from the frozen powdered rice roots and GS transferase activity was determined as described previously (Sakurai et al., 1996).

Re-introduction of OsGS1;2 cDNA into the knockout mutant

The entire 1,980 bp 5'-upstream region to the translation start codon of *OsGS1;2* was first fused with *OsGS1;2* cDNA in pDONR221, and then promoter-cDNA fusion gene was transferred into pGWB7, in order to add His6-tag codons and a stop codon (Nakagawa et al., 2007). Agrobacterium-mediated transformation was carried out using calli generated from wild-type Nipponbare and several independent lines of transgenic rice associated with the *OsGS1;2 promoter::OsGS1;2 cDNA*. After the self-fertilization, 10 independent lines possessing homozygous transgene at T2 generation were isolated. The transformants were then crossed with the homozygote of OsGS1;2 knockout mutant and the resulting seeds (F1) were harvested. These seeds were germinated and F1 plants were further self-fertilized and grown till maturity. After genotyping of the resulting F2 generation, independent three lines possessing homozygous *Tos17*-inserted *OsGS1;2* gene together with lines expressing *OsGS1;2 promoter::OsGS1;2 cDNA* were isolated. Because there was limitation on seed number of each line, two or three lines were used for the complementation tests.

Field trait and yield

Field traits and yield components were evaluated by observing the ripening stage of OsGS1;2 knockout mutants. Either no insertion null line (2009) or wild type of Nipponbare (2010) was used as a control. These rice plants were grown in paddy fields in 2009 and 2010, as described previously (Obara et al., 2004). Phenotypic measurements were performed as described by Tamura et al. (2010).

Statistics

Field traits and yield components were evaluated by observing the ripening stage of WT and three lines of NADH-GOGAT2 mutant grown in paddy fields in 2008 and 2009, as described by Obara et al. (2004). Phenotypic measurements were performed as described by Tamura et al. (2010).

Results

Isolation of mutants lacking GS1;2

A line of OsGS1;2 knockout mutants (line NF6488) was screened by searching the flanking sequence database (Miyao et al., 2003) of the mutant panel (http://tos.nias.affrc.go.jp/~miyao/pub/tos17/) of the Project for Rice Genome Research, where mutant lines, caused by the random insertion of endogenous retrotransposon *Tos17* into rice (*Oryza sativa*, L., cv Nipponbare) genome (Hirochika et al., 1996), were collected. In line NF6488, *Tos17* was inserted into exon-2 of the *OsGS1;2* gene from +377 to +323 where the translation start is +1 (Fig. 1a). The apparent abnormal order of nucleotide number was caused by the addition of identical 5 bp sequences (5'-*TTGAT*-3') at the right and left borders of *Tos17* in the NF6488 line, as seen in our previous studies on mutants of *OsGS1;1* (Tabuchi et al., 2005), *OsNADH-GOGAT1* (Tamura et al., 2010), and *OsNADH-GOGAT2* (Tamura et al., 2011). Reverse transcription-PCR showed that homozygous NF6488 line transcribed no *GS1;2* mRNA in roots, when the seedlings were grown for 18 days in nutrient solution containing 1 mM NH_4^+ (Fig, 1b). Total GS activity, assayed with a transferase activity, in the roots of the NF6488 line grown for 18 days in the presence of 1 mM NH_4^+ was less than a half of the activity in wild type roots (Fig. 1c).

Another seven independent lines (NF6484, NF6568, NF6584, NF6472, NF6572, and NF6588) were also isolated as candidate lines of OsGS1;2 knockout mutants using PCR screening against the mutant pool, but these lines showed no *Tos17* in *OsGS1;2* gene. Any mutant lacking GS1;2 was obtained, when possible candidate lines (PFG_1B-10506.R, and PFG_3D-00345.

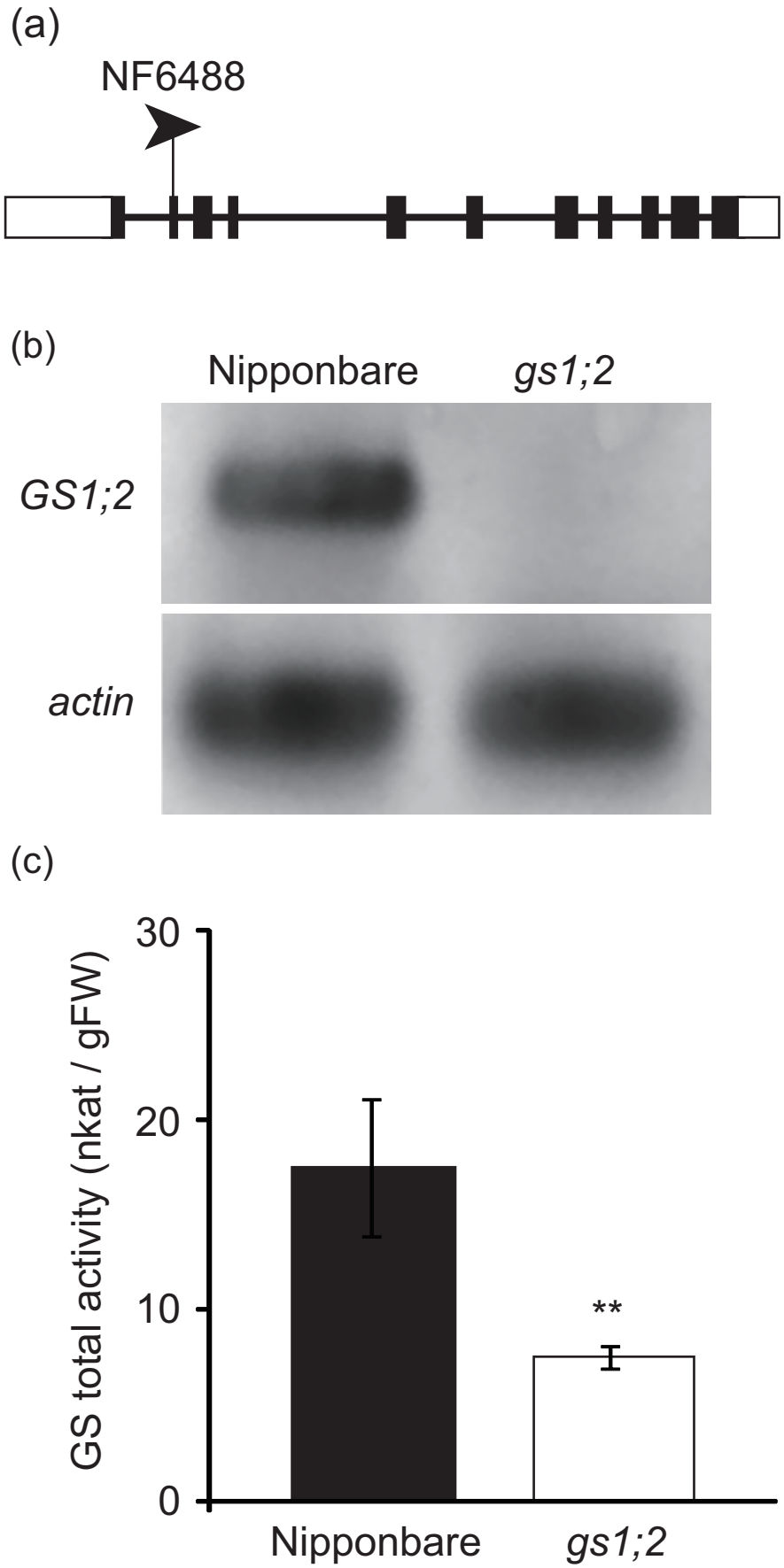


Figure 1. Isolation of OsGS1;2-knockout mutants of rice. (a) Diagram of the insertion position of the retrotransposon *Tos17* (arrowhead) in *OsGS1;2*. Exons are indicated as boxed regions whereas lines represent introns and the 5'- and 3'-untranscribed region. The open boxes correspond to untranslated sequences. (b) Confirmation of no expression of mRNA for *OsGS1;2* in a knockout line (NF6488) with RT-PCR. Wild-type Nipponbare and *Tos17*-inserted homozygous NF6488 (gs1;2) seedlings were grown hydroponically in the presence of 1 mM NH_4^+ for 18 days in a greenhouse with the roots harvested for RT-PCR analysis. (c) Total GS activity in roots of the knockout mutant. Crude extracts prepared from whole roots as described in (b) were used for GS transferase activity measurement. Means of independent triplicate samples and standard deviations (n = 3) are indicated. Significant differences between Nipponbare (filled column) and the mutant (opened column) identified by Student's *t* test are indicated with asterisks: $^{**}P < 0.01$.

R) in mutants, caused by random T-DNA insertion into genome of a rice cultivar Donjin (http://www.postech.ac.kr/life/pfg/risd/), were tested. Because only one GS1;2 mutant line was obtained, the phenotypic characteristics were compared with lines that re-introduced a full-length cDNA for *GS1;2* under the control of its own promoter, as described below.

Quantitative real-time PCR indicated that the homozygously inserted *GS1;2* NF6488 mutants expressed identical amounts of mRNAs for *OsGS1;1*, *OsGS2*, *OsNADH-GOGAT1*, *OsAMT1;2*, *OsAMT1;3* and *OsAS* in roots, when the GS1;2 mutant and wild-type Nipponbare of rice were grown hydroponically for 18 days in the presence or absence of 1 mM NH_4^+ (Fig. 2). Since mRNAs for *OsGS1;3* and *OsNADH-GOGAT2* were hardly detectable in rice roots (Tabuchi et al., 2007), these contents were not referred to the result. Thus, we succeeded in obtaining a knockout mutant lacking GS1;2.

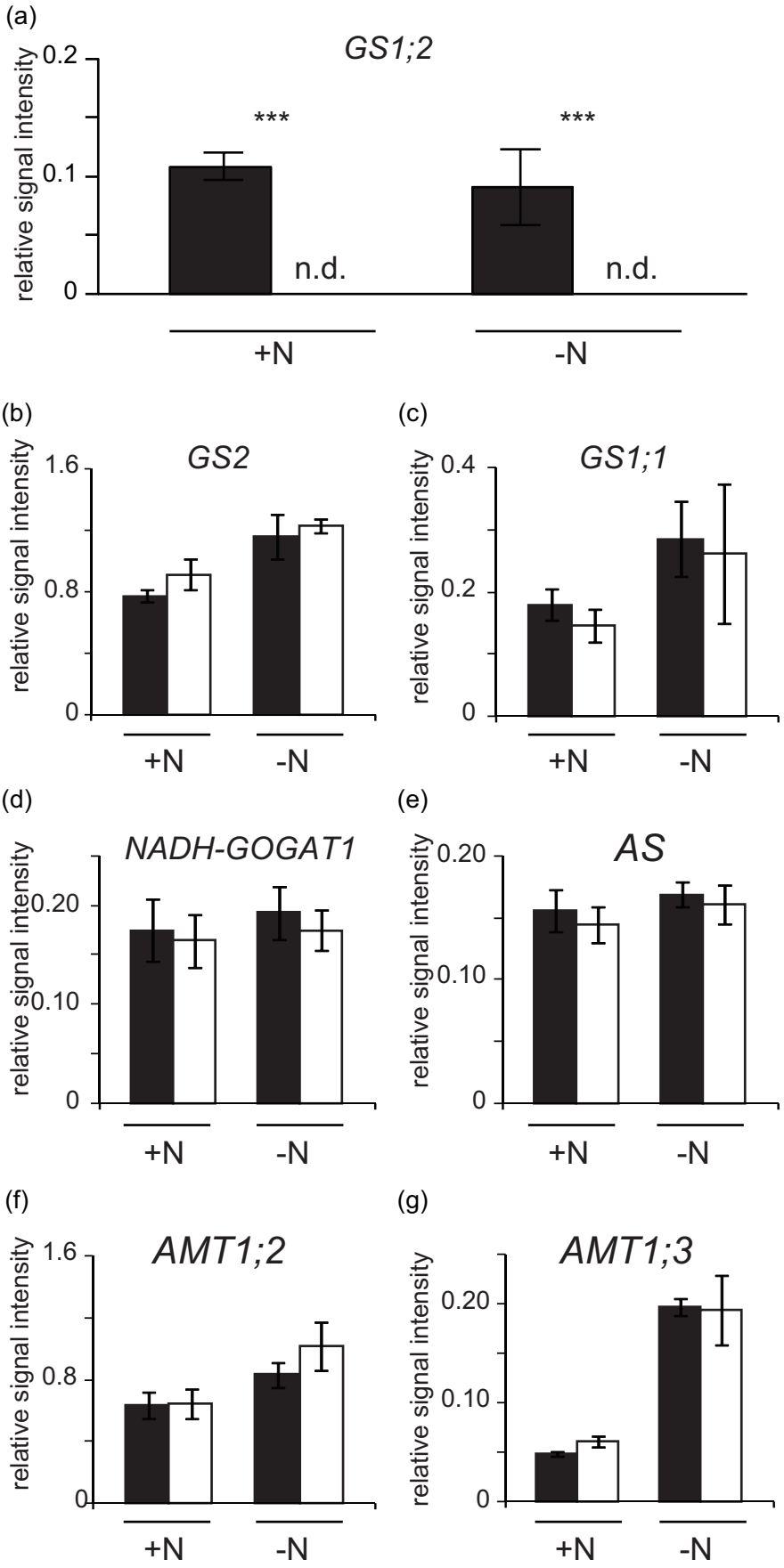


Figure 2. Real-time PCR detection of mRNAs for *OsGS1;2* (a), *OsGS2* (b), *OsGS1;1* (c), *OsNADH-GOGAT1* (d), *OsAS* (e), *OsAMT1;2* (f), and *OsAMT1;3* (g) from roots in a 18 day-old wild-type Nipponbare (filled column) and homozygote mutant (opened column) seedlings grown hydroponically in the presence (+N) or absence (-N) of 1 mM NH_4^+ for 18 days in green house. Contents of these mRNAs were normalized using actin mRNA, as described previously (Tamura et al., 2010). Means of independent triplicate samples and standard deviation (n = 3) are indicated. The "n.d." in (a) means "not detected". Significant differences between Nipponbare and the mutant identified by Student's *t* test are indicated with asterisks: ***$P < 0.001$.

Reduction of tiller number in OsGS1;2 knockout mutant grown till the harvest in paddy field

Field Trials were carried out in 2009 and 2010. No-insertion null plants, in which Tos17 had segregated out from the second exon of the OsGS1;2 gene during self-fertilization of the heterozygote NF6488, were used as a control. The phenotypic characteristics of the mutants obtained in 2009 were presented as Fig. 3. Reproducible results were obtained in 2009 and 2010. Tiller number was decreased by 50% in the mutants compared to the wild-type plants (Fig. 3a and 3b). Plant height was also decreased especially at vegetative stage of the OsGS1;2-knockout mutant. At the harvest, a severe reduction in panicle number was observed (Fig. 3c), although the panicle grain number (Fig. 3d), ratio of ripened spikelets (Fig. 3e), and 1,000-spikelet weight (Fig. 3f) was not significantly different between control and mutant plants. The reduction in panicle number caused severe decrease in yield of the mutant (Fig. 3g). Lack of GS1;2 also caused a marked decrease in shoot nitrogen content of rice plants at both vegetative (Supplemental Fig. 1a) and reproductive stages (Supplemental Fig. 1b). The results obtained in 2010 were shown as a Supplemental Fig. 2, where plants were treated with different supply of nitrogen into the field.

Since the *OsGS1;2* gene is mainly expressed in the surface cell layers of roots following

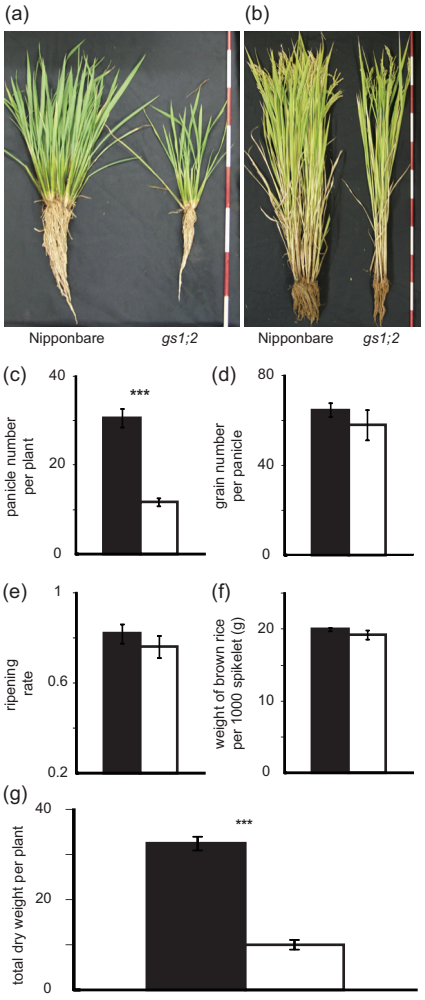


Figure 3. Phenotypic characteristics of OsGS1;2-knockout mutant (opened column in c to g) at vegetative stage (a, 42 days after planting) and at harvest stage (b to g, 137 days after planting) in a paddy field in 2009. A no-insertion null line (filled column), in which *Tos17* in *OsGS1;2* gene was fallen off during the self-fertilization, was used as a control plant. Yield components, such as panicle number per plant (c), main-stem grain number (d), proportion of well ripened grains (e), 1,000 brown rice weight (f), and total dry weight of rice plant (g), are indicated as the means of five independent samples with standard deviations (n = 5). Significant differences between Nipponbare and the mutant identified by Student's t test are indicated with asterisks: ***P < 0.001.

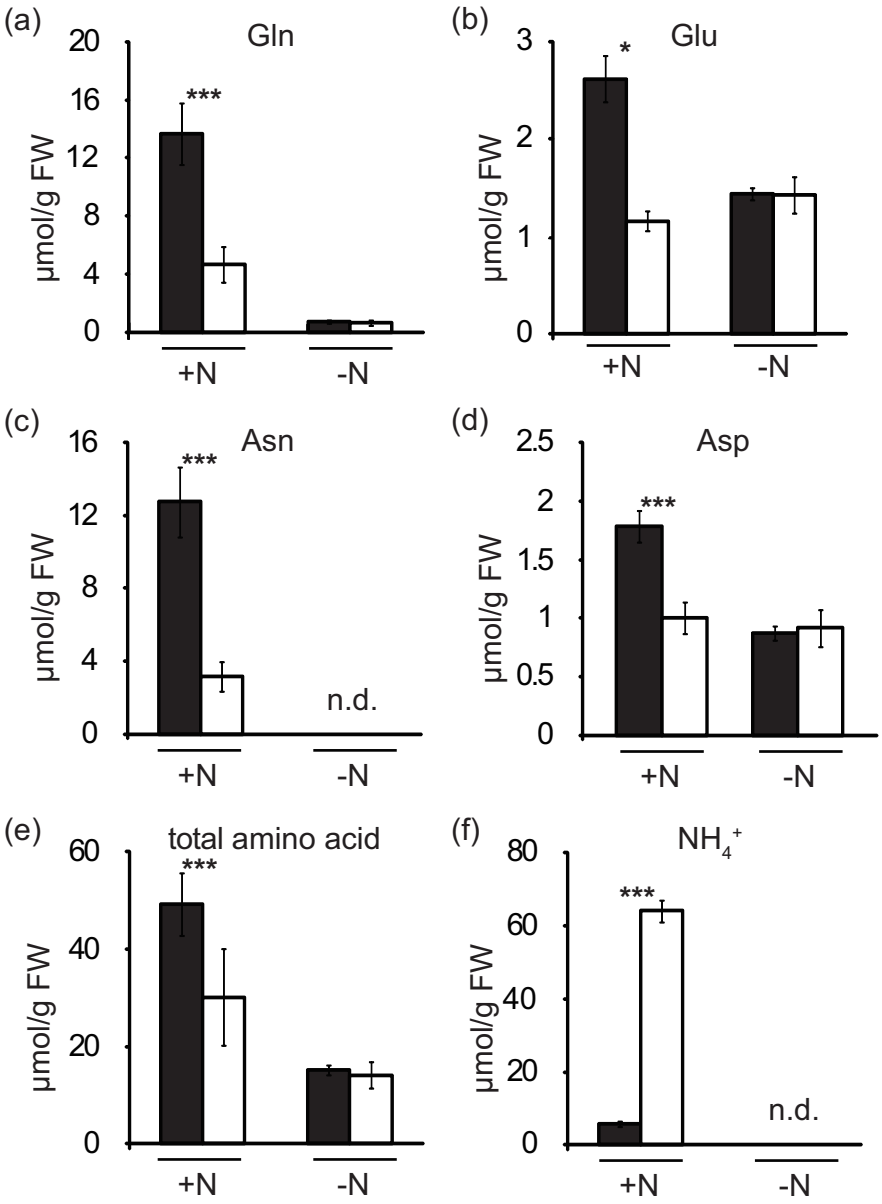


Figure 4. Comparison of free amino acids and NH_4^+ in roots of wild-type Nipponbare (filled column) with those of OsGS1;2-knockout mutant (opened column) grown hydroponically for 18 days in the presence (+N) or absence (-N) of 1 mM NH_4Cl.
Contents of Gln (a), Glu (b), Asn (c), Asp (d), total amino acids (e), and NH_4^+ (f) are indicated as µmol g-1 fresh weight of roots. Means of independent five samples and standard deviations (n = 5) are indicated. Significant differences between Nipponbare and the mutants identified by Student's *t* test are indicated with asterisks: *P<0.05, ***P < 0.001.

NH_4^+ supply at seedling stage (Ishiyama et al. 2004a, Tabuchi et al., 2007), contents of amino acids were determined in roots of both wild-type (*O. sativa* L. cv Nipponbare) and mutants at the seedling stage. As shown in Fig. 4, contents of glutamine, glutamate, asparagine, aspartate, and hence total amino acids were significantly reduced in the roots of the mutant, when the seedlings were grown in the presence of 1 mM of NH_4^+. In contrast, content of free NH_4^+ in the roots of the mutant increased more than seven-fold compared to that in the wild type roots. These changes were not observed, when the seedlings of wild type and the mutants were grown in the absence of NH_4^+. A significant reduction in amino acids and increase in free NH_4^+ were also detected in xylem sap collected from the mutant (Fig. 5). These results showed that the GS1;2 mutant apparently possesses a disorder on the primary assimilation of NH_4^+ in roots. This disorder seems to reduce the long distance transport of assimilated nitrogen

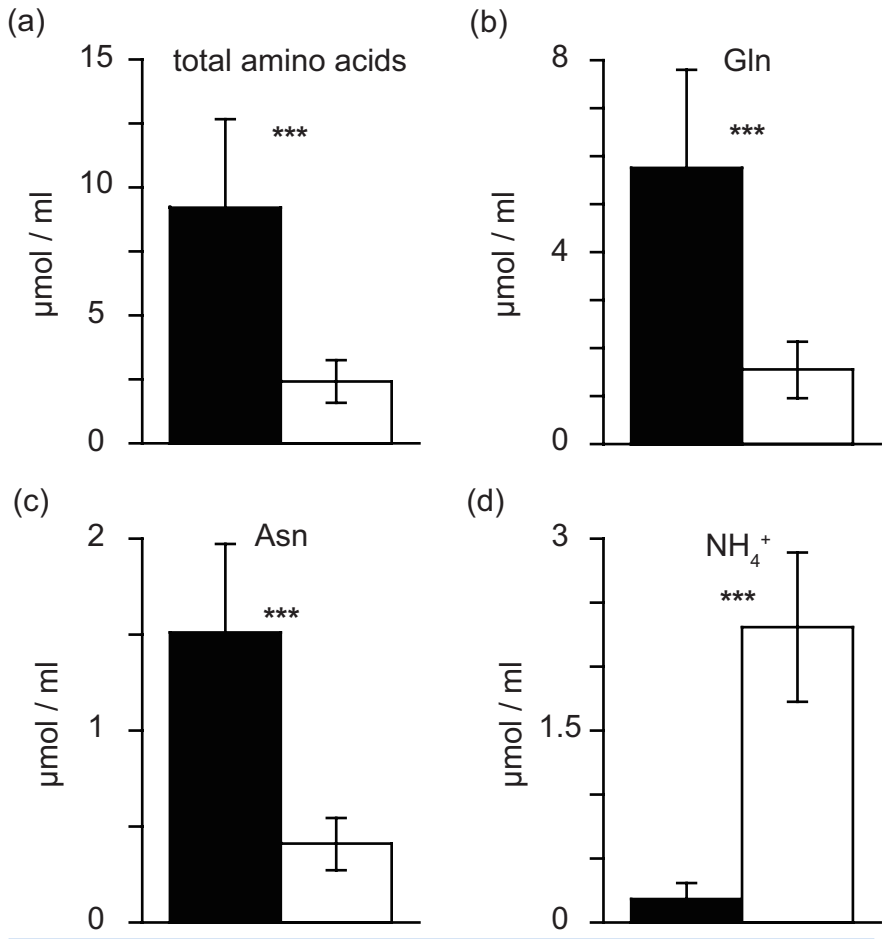


Figure 5. Comparison of free amino acids and NH_4^+ in xylem sap of wild-type Nipponbare (filled column) with those of OsGS1;2-knockout mutant (opened column) grown hydroponically for 35 days in the presence (+N) of 1 mM NH_4Cl. Contents of total amino acids (a), Gln (b), Asn (c), and NH_4^+ (d) are indicated as µmol ml-1 of xylem sap. Means of three independent samples and standard deviations (n = 3) are indicated. Significant differences between WT and the mutants identified by Student's *t* test are indicated with asterisks: ***$P < 0.001$.

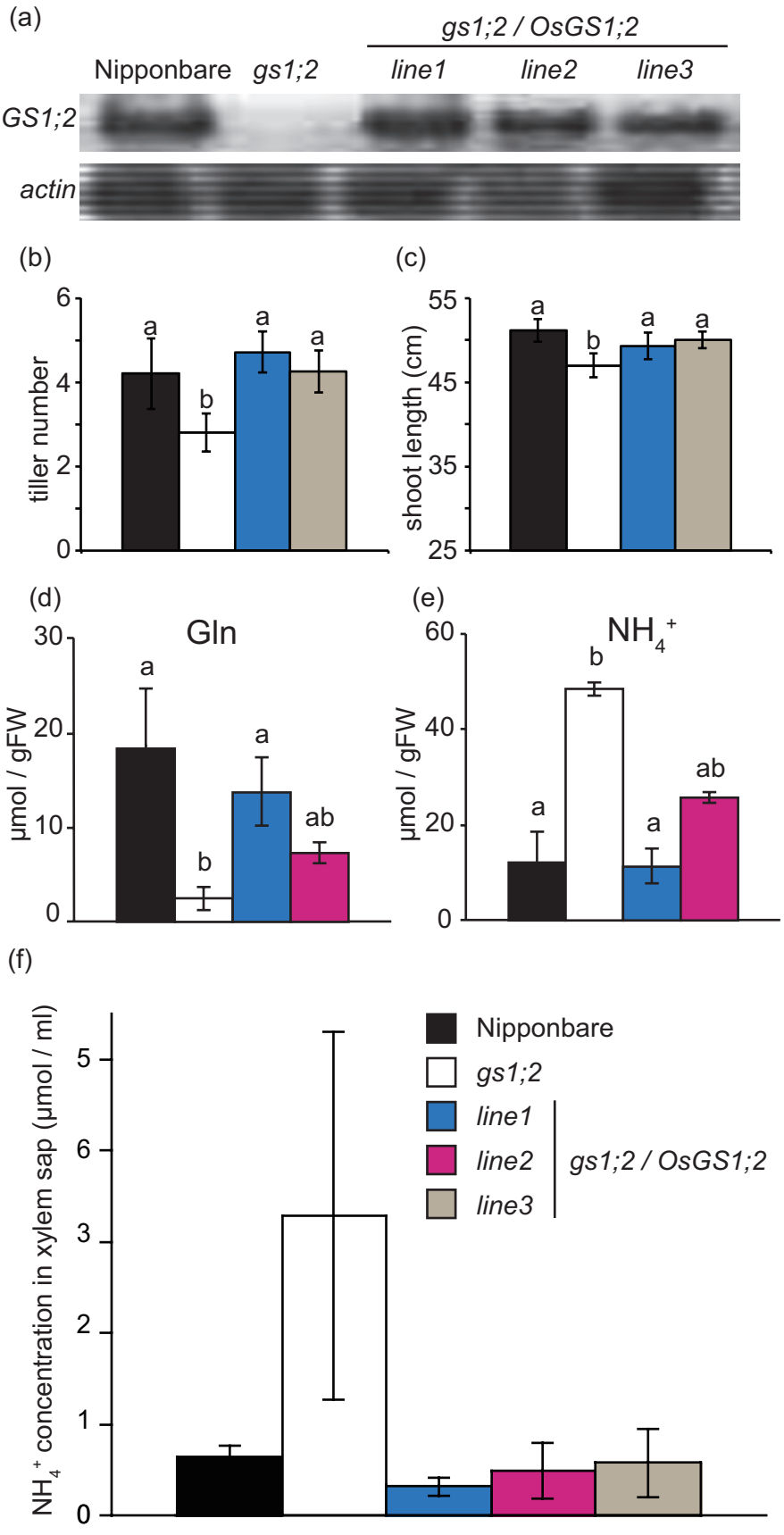
from the root to the top via xylem, and to affect the development of active tillers, panicle numbers, and productivity.

Complementation of GS1;2-knockout mutants by introducing OsGS1;2 cDNA

Although only one line of the GS1;2-knockout mutant was isolated in the current study, phenotypic and biochemical characteristics suggest that GS1;2 is important in the primary assimilation of NH4+ within roots. To confirm this function of GS1;2, a full-length cDNA for GS1;2 was introduced into homozygote mutants by crossing with an independent 10-lines of T1-generation transgenic Nipponbare overexpressing the cDNA under the control of its own promoter. The resulting F1 generation, possessing both the full-length cDNA and the *Tos-17* inserted *OsGS1;2* gene, were self-fertilized and grown until harvest. Three independent lines of the F2 generation inserted the homozygous *Tos-17* in *OsGS1;2* gene and *OsGS1;2* cDNA under the control of its own promoter were used for the complementation tests.

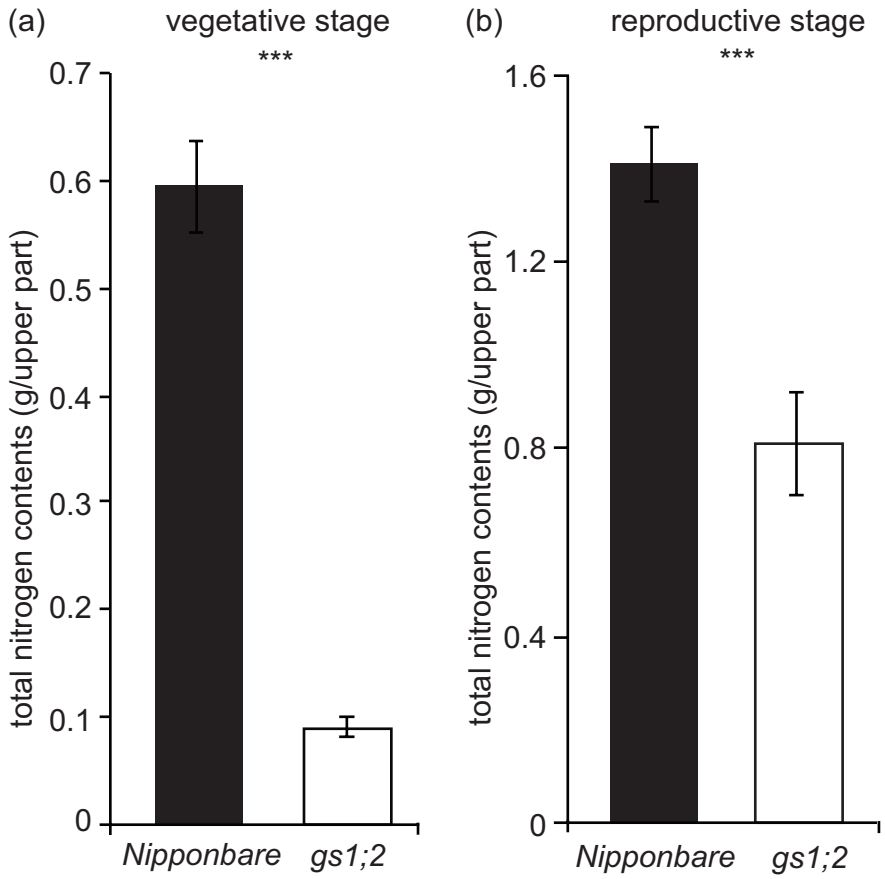
Phenotypic characteristics were tested using F2 generation plants grown in a greenhouse for 42 days in 1.3 l plastic pots with 1.0 g of slow-release fertilizer, as described by Tabuchi et al. (2005). Accumulation of mRNA originating from the transgene was confirmed by RT-PCR (Fig. 6a). Reductions in tiller number as well as the plant height, caused by the loss of functional GS1;2, were successfully complemented by the re-introduction of *OsGS1;2* cDNA into the knockout mutants (Figs. 6b and 6c). When the glutamine and NH_4^+ contents were compared in rice roots grown hydroponically for 18 days, free glutamine content in complementation lines was significantly higher than that of *Tos17* insertion line (Fig. 6d), while free NH_4^+ content was lower than that of *Tos17* insertion line (Fig. 6e), even though some fluctuation in these contents were seen in the complementation lines. When the xylem sap NH_4^+ concentration of 34 day old seedlings was measured, the increased NH_4^+ concentration seen in the knockout mutant was significantly reduced by the re-introduction of *OsGS1;2* cDNA (Fig. 6f). Thus, re-introduction of *OsGS1;2* cDNA into the knockout mutants successfully complemented the phenotypes.

Figure 6. Complementation of knockout mutants by introduction of OsGS1;2 cDNA.
Confirmation of transgene expression (*OsGS1;2 cDNA*) in the self-fertilized progeny of transformants with RT-PCR (a). Comparison of tiller numbers (b), shoot length (c), glutamine in root (d), NH_4^+ in root (e) and NH_4^+ in xylem sap (f) were determined using wild-type Nipponbare (black column), OsGS1;2-knockout mutants (white column), and two or three complementation lines (blue, red, or brown column) produced by crossing of T2 plants containing the homozygous transgene, *OsGS1;2 promoter::OsGS1;2 cDNA*, with the OsGS1;2-knockout mutants, and then self-fertilized. All lines were grown hydroponically for 35 days. Means of three to eight independent samples and standard deviations are indicated in (b) to (f). Significant differences in these three lines identified by one-way ANOVA are indicated by lower-case letters (a and b) on error bars.



Discussion

Current studies with knockout mutants have significantly extended our understanding of physiological function of GS1;2 in rice plants. Knockout of the *OsGS1;2* gene caused a marked decrease in the active tiller number associated with a reduction in free amino acid content, and the enhancement of free NH_4^+ concentration in hydroponically grown vegetative rice roots. This phenotype was complemented successfully by introduction of *OsGS1;2* cDNA under the control of its own promoter. Mutants showed a severe reduction in panicle number per plant, resulting an approximately 60% decrease in yield, when the mutants were grown until harvest. Rice yield is defined as the product of panicle number, spikelet number, proportion of well ripened grains, and spikelet weight (Sakamoto and Matsuoka, 2008). Disruption of *OsGS1;2* gene showed little or no effect on the spikelet number, proportion of well ripened grains, and spikelet weight. This phenotype is quite similar to N-deficiency symptoms in rice (Mae, 1997). Recent literatures pointed the dependency of tiller numbers on a phytohormone, strigolactone (Luo et al., 2012). It is known that some of the action of strigolactone is tightly related to nutrient conditions, such as Pi-supply (Seto et al., 2012). Therefore, it is possible to hypothesize that the loss of GS1;2 may decrease the concentration of strigolactone in knockout mutant. Since *OsGS1;2* gene was expressed predominantly in roots grown in the presence of NH_4^+ (Tabuchi et al., 2007), the marked decrease in the panicle number is probably caused by the shortage of nitrogen that is transported from the roots throughout growth. As shown in Figs. 4 and 5, increase in free NH_4^+ in both roots and xylem sap probably indicates that some portion of NH_4^+,

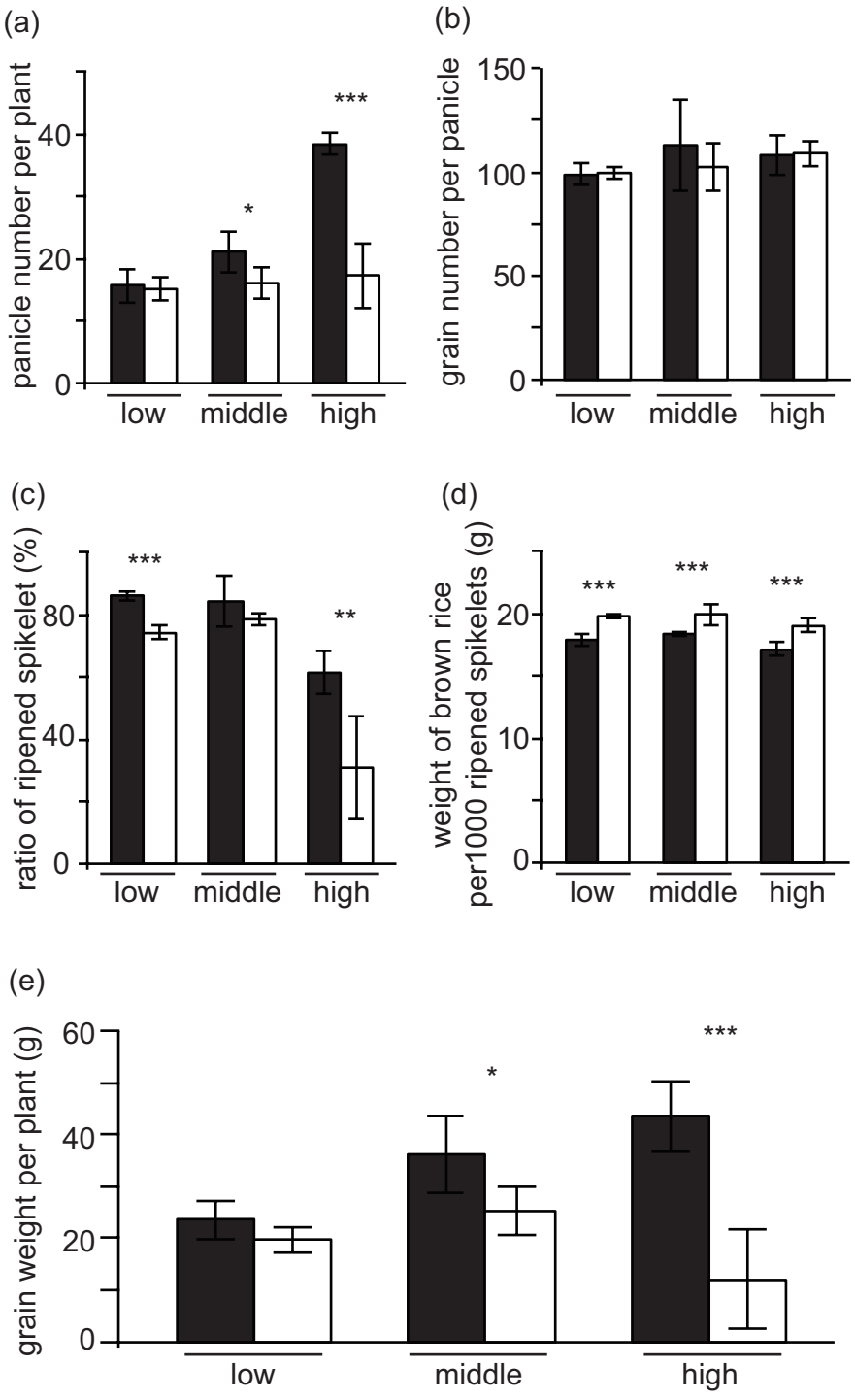


Supplemental Figure 1. Total nitrogen contents in top part of wild-type Nipponbare (WT, filled column) and OsGS1;2-knockout mutant (opened column) at the vegetative (a) and reproductive stages (b). Rice plants were grown in a paddy field under "Normal" N conditions, as reported in the legend of supplemental Figure 1, in 2010 and whole top parts were harvested either at 54 days (vegetative stage) or 111 days (reproductive stage) after transplanting. Means of five independent samples and SD (n = 5) are indicated. Significant differences between WT and the mutants identified by Student's t test are indicated with asterisks: ***P < 0.001.

not assimilated within the roots, could be transported to the top parts and assimilated in leaves. This could be a reason why the mutants were not lethal.

Within the roots, the OsGS1;2 and OsNADH-GOGAT1 genes were expressed abundantly in two cell-layers of the root surface (Ishiyama et al., 2003, 2004a). Based on these localization studies, we hypothesized that GS1;2 and NADH-GOGAT1 could be key players in the assimilation of NH4+ taken up by rice roots (Tabuchi et al., 2007). Supporting this hypothesis, OsGS1;2 mutants at the seedling stage showed a marked decrease in contents of free amino acids, especially glutamine, asparagine, glutamate, and aspartate when grown with 1 mM NH4+. Conversely, free NH4+ content increased approximately 10-fold, when compared with wild-type roots. Since asparagine is synthesized from glutamine by the reaction of asparagine synthetase (Lea et al., 2007) and glutamate from glutamine by the reaction of NADH-GOGAT1 (Tabuchi et al., 2007), the decrease in the glutamine content is apparently caused by the lack of GS1;2 in the mutants. Decreases in glutamine and asparagine contents as well as the increase in NH4+ content were also detected in xylem sap, indicating that the mutants are less efficient in the long distance transport of assimilated nitrogen from the roots to the leaves. As these phenotypes could be successfully recovered by the re-introduction of OsGS1:2 cDNA, we conclude that GS1;2 is crucial in the primary assimilation of NH4+. Although OsGS1;1 was also expressed in rice roots (Tabuchi et al., 20005, 2007), the GS1;1 was not able to fully compensate for GS1;2 function. Our recent studies with OsNADH-GOGAT1 mutants also showed a reduction in panicle number per plant, with NADH-GOGAT1 providing glutamate to GS1;2 for the assimilation of NH4+ (Tamura et al., 2010).

As far as we know, this study, together with that of Tamura et al. (2010), is the first instance to identify the molecular species of GS1 and NADH-GOGAT that are responsible in the primary assimilation of NH4+ in plants. Studies with mutants have been applied to characterize GS2 (Wallsgrove et al., 1987) and Fd-GOGAT (Kendall et al., 1986) in barley under photorespiratory conditions. GS2 mutants in Lotus japonicas resulted in profound limitations in carbon metabolism that affect the nodulation process and nitrogen fixation (Garcia-Calderon et al., 2012). The importance of GS1-4 in kernel size and GS1-3 in kernel number of maize plants was reported by Martin et al. (2006), similar to the case of GS1;1 knockout in rice where GS1;1 is important for normal growth and grain filling (Tabuchi et al., 2005). Our recent studies with OsNADH-GOGAT1 knockout mutants demonstrated the importance of NADH-GOGAT1 in both primary assimilation in roots and the development of active tiller number in rice (Tamura et al., 2010). Although there is only one NADH-GOGAT gene in Arabidopsis thaliana, the importance of NADH-GOGAT in non-photorespiratory metabolism has been indicated using glt1-T mutants (Lancien et al., 2002). Thus, previous observations are not directly related to the primary assimilation of NH4+. Less attention has been paid to the assimilation of NH4+ in



Supplemental Figure 2. Phenotypic characterization of the mutant at harvest stage cultivated in paddy field in 2010. Wild-type Nipponbare (filled column) and OsGS1;2-knockout mutant (opened column) were grown until harvest in paddy field supplied with either no fertilizer (Low N), 30 kg per hectare of slow-released fertilizer consisting of 16% (w/w) of N, P, and K (TEMAIRAZU, Co-op Chemicals, Tokyo, Japan) (Normal N), or Normal N supplemented with 30 to 60 kg of (NH4)2SO4 per hectare every two weeks (270 kg in total) (High N) for 144 days after transplanting, as described by Obara et al. (2004). Panicle number per plant (a), grain number per panicle on a main stem (b), proportion of well ripened grains (c), 1,000-brown rice weight (d), and total weight of brown rice per plant (e) were measured with five independent samples. Means and SD are indicated. Significant differences between WT and the mutants identified by Student's t test are indicated with asterisks: *P < 0.05, **P < 0.01, ***P < 0.001.

roots compared with the assimilation of other forms of nitrogen probably due to the uniqueness of rice plants which grow predominantly in anaerobic paddy field conditions. Unlike upland cropping, NH4+ is the major nitrogen source for rice. Most upland plants use NO3- that are primarily reduced and assimilated in leaf tissues after the transport from roots via the xylem (Yamaya and Oaks, 2004). Therefore, rice plants have apparently evolved characteristics for the efficient uptake and assimilation of NH4+ both in terms of their metabolism and root morphology (see: Tabuchi et al., 2007). For example, rice plants possess specialized morphology of roots in order to grow in water by means of development of cortical air space (aerenchyma) for oxygen supply from the top parts, as well as development of a Casparian strip between the second (exodermis) and the third cell layers (scrlenchyma) of the root surface (Morita et al., 1996) that is probably for the prevention of oxygen leakage from the roots, in addition to a Casparian strip in endodermis cells seen in most of plants. Since GS1;2 and NADH-GOGAT1 are located in epidermis and exodermis, most NH4+ could be assimilated within these cell types and the resulting glutamine or glutamate are symplastically transported across the Casparian strip into cortex cells of the roots. Lack of GS1;2 could result in less efficient assimilation of NH4+ and hence decreased a development of active tillers. It is worth noting that these two cell types of rice roots also contain GS1;1, but its expression rapidly decreases following NH4+ supply (Ishiyama et al., 2004a, Tabuchi et al., 2007). This is in contrast to the rapid up-regulation of OsGS1;2 gene. We have no immediate answer why GS1;1 does not compensate for GS1;2 functions in the knockout mutants, but independent regulatory systems probably occur for the expression of OsGS1;1 and OsGS1;2 genes in rice roots. Metabolomics data using GS1;1-knockout mutants showed that

GS1;1 possesses a crucial function in coordinating metabolic function in rice plants (Kusano et al., 2011). Works on regulation of gene expressions for GS1;1 and GS1;2, and profiling of transcripts and metabolites in those mutants are now in progress to answer questions on the complex network of nitrogen metabolism in rice. Although long life cycle of rice is a disadvantage compared to other model plants, a new breeding technique recently developed by Ohnishi et al. (2011) could be a valuable method for genetic studies in rice.

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