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Reverse genetics approach to characterize a function of NADH-glutamate synthase1 in rice plants

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Rice plants grown in anaerobic paddy soil prefer to use ammonium ion as an inorganic nitrogen source for their growth. The ammonium ions are assimilated by the coupled reaction of glutamine synthetase (GS) and glutamate synthase (GOGAT). In rice, there is a small gene family for GOGAT: there are two NADH-dependent types and one ferredoxin (Fd)-dependent. Fd-GOGAT is important in the re-assimilation of photoresiratory generated ammonium ions in chloroplasts. Although cell-type and age dependent expression of two NADH-GOGAT genes has been well characterized, however, metabolic function of individual gene product is not fully understood. Reverse genetics approach is a direct way to characterize functions of isoenzymes. We have isolated a knock-out rice mutant lacking NADH-dependent glutamate synthase1 (NADH-GOGAT1) and our studies show that this isoenzyme is important for primary ammonium assimilation in roots at the seedling stage. NADH-GOGAT1 is also important in the development of active tiller number, when the mutant were grown in paddy field until the harvest. Expression of NADH-GOGAT2 and Fd-GOGAT in the mutant was identical with that in wild-type, suggesting that these GOGATs are not able to compensate for NADH-GOGAT1 function.

Introduction

The form of inorganic nitrogen that is available for the growth of rice plants in paddy soil is the ammonium ion (NH_4^+). The NH_4^+ is taken up by rice roots by high affinity-type NH_4^+ transporters (Sonoda et al. 2003). It is then assimilated into the amide residue of glutamine (Gln) by the coupled reaction of glutamine synthetase (GS) and glutamate synthase (GOGAT). The GS catalyzes an ATP-dependent conversion of glutamate (Glu) to Gln using NH_4^+, whereas GOGAT generates two molecules of Glu from Gln and 2-oxoglutarate using reduced ferredoxin (Fd) or NADH as a reductant. It is now well established that the GS/GOGAT cycle is the only route for the primary assimilation of NH_4^+ in plants grown under normal conditions (Ireland and Lea 1999; Lea and Miflin 2003). The major forms of nitrogen in xylem sap of rice plants are Gln and asparagine (Asn) (Fukumorita and Chino 1982). Real-time monitoring of NH_4^+ transport from root to shoot of rice by the positron emitting tracer imaging system showed that the signals of ^{13}N taken up by rice roots were detected in the basal part of shoots within a short period, but the transport was completely inhibited by methionine sulfoximine, an inhibitor of GS (Kiyomiya et al. 2001). These results suggest that most of the NH_4^+ taken up by the roots can be assimilated within the roots. Excess NH_4^+ can apparently be toxic to some plants (Kronzucker et al. 2001). Thus, efficient NH_4^+ uptake as well as the subsequent assimilation systems seems highly regulated within the roots.

In the top part of rice, approximately 80% of the total nitrogen in the panicle arises from remobilization from senescing organs (Mae and Ohira 1981). The remobilization occurs through phloem and the major forms of nitrogen in the phloem sap of rice are Gln and Asn (Hayashi and Chino 1990). Therefore, Gln should be synthesized from the catabolic products of proteins, nucleic acids, and chlorophyll, while Asn is synthesized from Gln (Lea et al. 2007). After Gln reaches the developing sink organs, it is converted via GOGAT reaction into Glu, which is a central amino acid for the synthesis of a number of amino acids (Ireland and Lea 1999). This is mostly responsible for the metabolism of Gln in rice (Tobin and Yamaya 2001). In addition, overlapping expression of genes for GS and phenylalanine ammonia lyase, which is the key enzyme in the phenylpropanoid metabolism, was detected in vascular bundles of developing young leaf blade of rice (Sakurai et al. 2001). These observations suggest that NH_4^+ generated from the catabolic processes during senescence as well as the secondary metabolism is re-assimilated via GS / GOGAT reactions.

In rice, there are three genes for cytosolic GS, i.e. *OsGS1;1*, *OsGS1;2* and *OsGS1;3*, and one gene for chloroplastic

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GS, *OsGS2* (Tabuchi et al. 2007). Also, there are two genes for NADH-GOGAT, i.e. *OsNADH-GOGAT1* and *OsNADH-GOGAT2*, and one gene for *Fd-GOGAT* (Tabuchi et al. 2007). The GS2 and Fd-GOGAT are in the chloroplasts of green tissues of rice (Hayakawa et al. 1994; Sakurai et al. 1996). The major function of GS2 and Fd-GOGAT is in photorespiratory nitrogen metabolisms (Lea and Miflin 2003). The cytosolic GS1 and NADH-GOGAT are important for the normal growth and development (Yamaya and Oaks 2004), since mutants lacking either GS2 or Fd-GOGAT were able to grow normally under non-photorespiratory conditions (Kendall et al. 1986; Wallsgrove et al. 1987). It has been shown that the expression profile of three GS1 genes and two NADH-GOGAT genes was different in term of cell-type and age specificity, and in response to exogenous NH_4^+, suggesting that each gene product has a distinct function in rice (Tabuchi et al. 2007).

Reverse genetics approach is powerful for elucidating gene function. In our previous work, we showed the characteristics of *OsGS1;1*-knockout mutants (Tabuchi et al. 2005) generated by the insertion of an endogenous retrotransposon *Tos17*. Recently, we were successful in isolating *OsNADH-GOGAT1* knockout mutant from the mutant pool. Here, we discuss possible function of NADH-GOGAT1.

Materials and Methods

Plant materials

Rice lines (NG6590, NG0080 and NG0088) were screened by searching the flanking sequence database of the mutant panel (http://tos.nias.affrc.go.jp/~miyao/pub/tos17/) of the Project for Rice Genome Research, where mutant lines are generated by the random insertion of endogenous retrotransposon Tos17 into rice genome (Hirochika et al. 1996). For studying the expression of *OsNADH-GOGAT1* mRNA and accumulation of its protein, the seedling of WT (Nipponbare), and *Tos17*-inserted homozygous NG6590 and NG0088 lines were first grown in tap water (pH 5.5) for 2 weeks and then on 1/4th strength of hydroponic solution without nitrogen for 3 days (Mae and Ohira, 1981). Some of the seedlings were further grown for three or 24 hours in the presence of 1 mM NH_4Cl and whole roots were harvested for the analyses. Genomic DNA was extracted from two-week old seedlings for determination of genotype, as well as the position of *Tos17* insertion into *OsNADH-GOGAT1* gene by PCR and sequencing as described below. When effects of various concentrations NH_4Cl on the growth phenotype and contents of amino acids and plant hormones were determined, seeds of WT and NG0088 were first selected by soaking in NaCl solution (d=1.13) with gentle shaking. The seeds that sank to the bottom were washed with tap water for 20 minutes and dried at room temperature. The dried seeds were sterilized with 1% (v/v) sodium hypochlorite solution for 20 minutes and then washed with running tap water. The seeds were germinated in distilled water at 30 °C in the dark for 36-40 hours. The germinated seeds were sown on a nylon net floated on 1/4th strength hydroponic solution containing 0-5000 μM NH_4Cl or 1 mM KNO_3 as a sole source of nitrogen in an outdoor growth chamber. The temperature was controlled at 26°C from 5:00 to 19:00 with supplemental light during the day and at 23°C from 19:00 to 5:00 during the night. The culture solution was renewed every day from day 2 and the roots were harvested six days after sowing. Under field conditions, WT, NG0088 mutant line and no-insertion null mutant seeds were first germinated and sown on a synthetic culture soil as described by Obara et al. (2004). These plants were grown in paddy field in Kashimadai, Miyagi, Japan, till the ripening stage and harvested, as described by Obara et al. (2004).

RT-PCR and quantitative real-time PCR

Total RNA was extracted from the roots at 3 hours after supplying NH_4Cl by using RNeasy Plant Mini Kit (QIAGEN, Hilden, Germany). PrimeScript® RT reagent Kit (TAKARA BIO INC. Otsu, Japan) was used to synthesize the first-strand cDNA. Reverse transcription (RT)-polymerase chain reaction (PCR) analysis for NG0088 line was carried out using OsNADH-GOGAT1 specific primers as follows: forward primer, 5' - ACC CTG GAA TGA TGC TG T TG - 3' and reverse primer, 5' - ATA GCT CCC CGA GCT TCT GT - 3'. A set of primers, 5' - TGT GGA TGC TAC CTC GTC TG - 3' (forward) and 5' - TGT GGA TGC TAC CTC GTC TG - 3' (reverse), was used when expression of OsNADH-GOGAT1 in NG6590 line was determined with RT–PCR. Forward primer, 5' - GGC TGG AAT TGC TCT TAA C - 3' and reverse primer, 5' - CAG CAT AGA CAA AGC ATA CC - 3' were used for the detection of OsGAPDH mRNA. PCR amplification and electrophoresis were carried out as described by Tabuchi et al. (2005). Quantitative real-time PCR analysis was carried out using gene-specific primers as follows: forward primer, 5' - GTG CAG CCT G TT GCA GCA TAA A - 3' and reverse primer, 5' - CGG CAT TTC ACC ATG CAA ATC - 3' for *OsNADH-GOGAT1* mRNA, as described by Tabuchi et al. (2007). Forward primer, 5' - GCA TAC TTG TGA AGC ACC GAA GTG - 3' and reverse primer, 5' - CTG CAA ATA GCA ACC TAG CGT CAG - 3' were used for determination of OsFd-GOGAT mRNA. Gene-specific primers for *OsNADH-GOGAT2*, *OsGS1;1*, and *OsGS1;2* were the same as described by Tabuchi et al. (2007). Primers for *OsGDH1* encoding glutamate dehydrogenase (GDH) subunit gene and actin were used as described by Abiko et al. (2005). The PCR products were detected by SYBR® Premix Ex TaqTM II (TAKARA BIO INC.) using Light Cycler® 480 (Roche Diagnostics Corp.,

Tokyo, Japan).

Immunoblotting

Plant tissues were homogenized in 100 mM HEPES-NaOH (pH 7.5), 0.2% (v/v) 2-mercaptoethanol, 1 mM EDTA, 1 mM phenylmethylsulfonylfluoride, 10 µM leupeptin, 500 µM 4-(2-amino-ethyl)-benzenesulfonyl fluoride, 10% (v/v) glycerol and 0.2% (v/v) Triton X-100 [3 ml/g FW] as described by Abiko et al. (2005). The crude protein fraction was prepared from the homogenate, proteins separated by SDS-PAGE, transferred on to a membrane and immunoblotting was done with either the anti-NADH-GOGAT IgG to detect NADH-GOGAT1 protein or anti-GS1 IgG to detect GS1 protein, as described previously (Yamaya et al., 1992; Ishiyama et al., 2004).

Measurement of free amino acid concentration

Four or five replicates of independent seedling roots were used for measurement of free ammonium and amino acid concentrations. Frozen root sample (9.2-31.8 mg) were powdered in liquid N2 and then homogenized in 10 volumes (10 µl for 1 mg sample) of 10 mM HCl. Extraction for and determination of amino acid concentrations were as described by Tabuchi et al. (2005) with minor modifications. Separation module used was Waters 2695 with AccQ Taq column (Waters Corp., Milford, U.S.A).

Hormone analysis

Roots (58.9-107.9 mg fresh weight) of WT or *OsNADH-GOGAT1* (NG0088) seedlings were prepared in a microcentrifuge tube. Profiling of plant hormones and their derivatives were determined as described by Kojima et al. (2009).

Field trait and yield

Traits and yield components were evaluated using the ripening stage of WT, *OsNADH-GOGAT1* mutant (-/-) and null mutant (+/+) grown in paddy field. Phenotypic measurements were performed for 8 traits (n = 5 to 10): Top dry weight per plant and panicle dry weight per plant were measured after the samples were dried to a constant weight at 80°C. Weight of brown rice per plant was counted as an average of total spikelet per plant. Ripened spikelet number per plant was determined as an average of ripened grain number selected by soaking of the seeds in a NaCl solution (d=1.06). Panicle number per plant was counted as an average of panicles per plant. Weight of brown rice per 1,000 spikelets was obtained as an average of 1,000 spikelet weight from each plant. Spikelet number per panicle was counted as an average of spikelets per total panicle number of a plant. Ratio of ripened spikelet was calculated as the ratio ripened spikelet number per total grain number.

Results

Isolation of knockout mutants for *OsNADH-GOGAT1* in rice

Lines of *OsNADH-GOGAT1* knockout mutants (lines NG0080 and NG0088) have a *Tos17* insertion at the same position in exon 13 (from +3,760 to +3,756 when the translation start is +1) (Fig. 1a). The

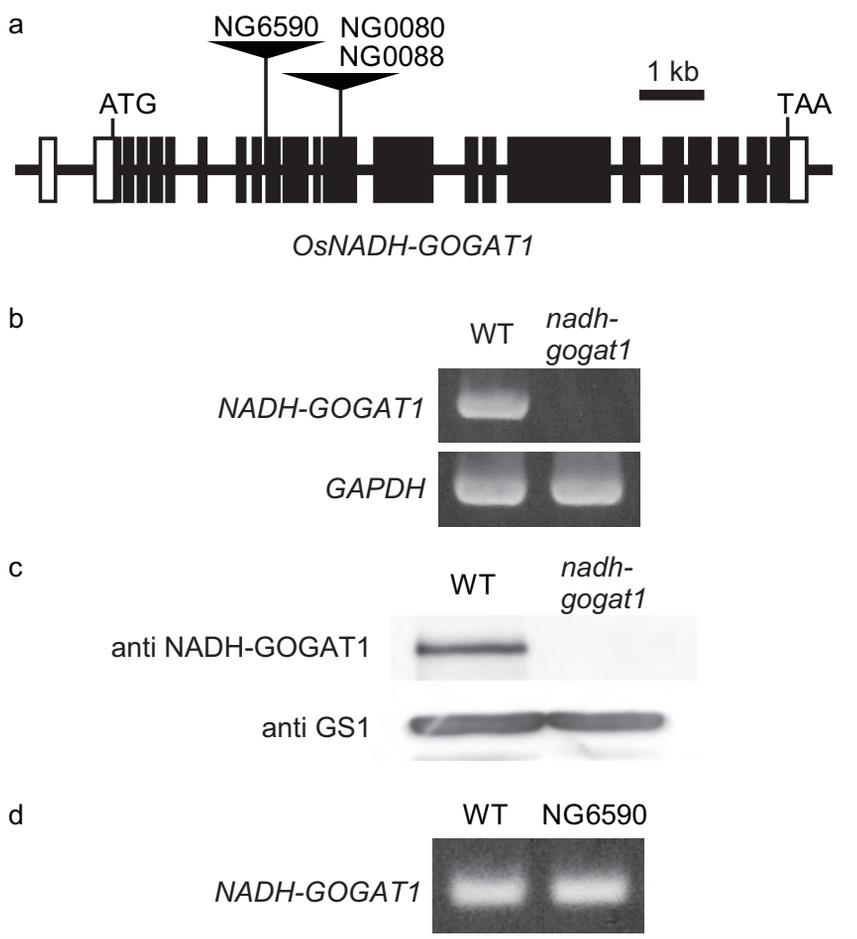

Figure.1 Position of *Tos17* inserted into *OsNADH-GOGAT1* gene and expression of *OsNADH-GOGAT1* in retrotransposon *Tos17* insertion lines. (a) Diagram of insertion position of retrotransposon *Tos17* in *OsNADH-GOGAT1*. Exons are indicated as boxed region whereas lines represent introns and the 5'- and 3'-untranscribed regions. (b) RT-PCR detection of *OsNADH-GOGAT1* mRNA from roots in 26-day-old seedling of wild type (WT) and homozygote NG0088 line (*nadh-gogat1*) following the supply of 1 mM NH_4Cl for three hours. Glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*) mRNA was determined in these roots as a control (right panel). Means of independent triplicate samples and standard deviation values (n=3) are indicated. (c) Immunodetection of NADH-GOGAT1 protein in extract from roots of WT and homozygote NG0088 line (*nadh-gogat1*) following the supply of 1 mM NH_4Cl for 24 hours. GS1 protein was used as a control. (d) RT-PCR detection of *OsNADH-GOGAT1* mRNA from roots of WT and another homozygote NG6590 line in which *Tos17* is inserted in intron 9.

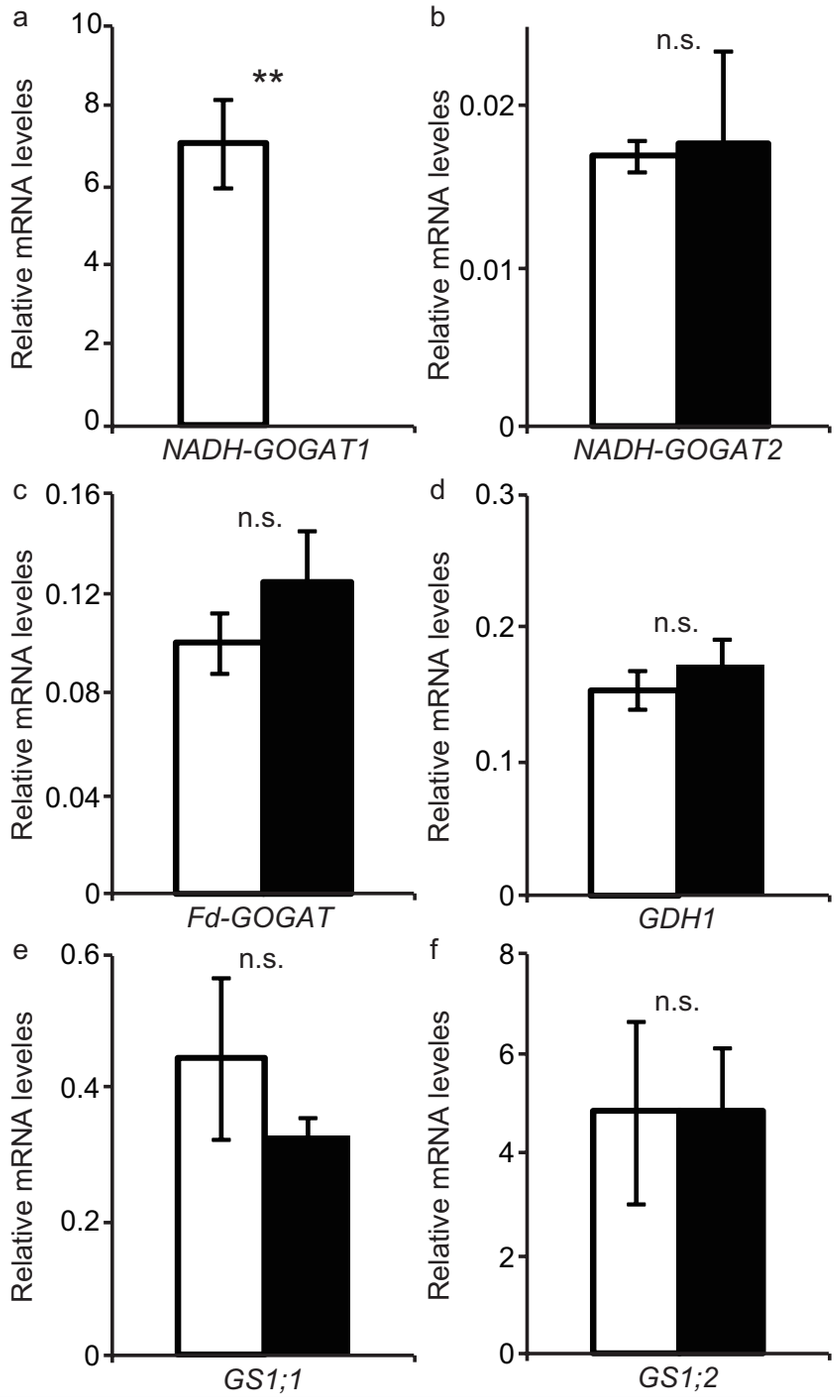

Figure. 2 Real-time PCR detection of mRNAs for *OsNADH-GOGAT1* (a), *OsNADH-GOGAT2* (b), *OsFd-GOGAT* (c), *OsGDH1* (d), *OsGS1;1* (e), and *OsGS1;2* (f) from roots in a 26-day-old seedling of wild-type (open column) and homozygote NG0088 line (black column) following the supply of 1 mM NH_4Cl for 3 h. Contents of these mRNAs were normalized using actin mRNA. Means of independent triplicate samples and standard deviation values (n = 3) are indicated. The "n.s." above each panel means "not significantly different"

apparent abnormal order of nucleotide number was caused by the identical sequences at the right and left borders of *Tos17*, as seen in the previous studies with *OsGS1;1* mutants (Tabuchi et al. 2005). Thus, line NG0088 was used for further investigation. In our previous work, we showed that mRNA for NADH-GOGAT1 in roots of rice seedlings transiently accumulated following the supply of 1 mM NH_4^+ (Hirose et al. 1997; Ishiyama et al. 1998; Tabuchi et al. 2007). Therefore, expression of *OsNADH-GOGAT1* gene in lines NG0088 and NG6590 was determined using the conditions of NH_4^+-dependent accumulation in wild type as described in the Materials and Methods. Quantitative real-time PCR showed that the NG0088 line transcribed no detectable mRNA for NADH-GOGAT1 in roots when the seedlings were grown for three hours in the presence of 1 mM NH_4^+ (Fig. 1b). Similarly, NADH-GOGAT1 protein was not detected in the roots (Fig. 1c). On the other hand, mRNA for NADH-GOGAT1 was detected in the wild-type when NG6590 line was tested using RT-PCR methods (Fig. 1d). The NG0088 line has been found to show no significant changes in the expression of mRNAs for *OsNADH-GOGAT2*, *OsFd-GOGAT*, *OsGS1;1*, *OsGS1;2* and *OsGDH1* under the same growth conditions (Fig. 2). Thus, we succeeded in obtaining a specific knockout mutant for *OsNADH-GOGAT1*. Because only one line of the mutant was isolated, the phenotypic characteristics at the harvest were compared with our preliminary results obtained with transgenic lines over-expressing OsNADH-GOGAT1 gene in an indica cultivar, Kathalath (Yamaya et al. 2002).

Characteristics of OsNADH-GOGAT1 mutant at the seedling stage

The OsNADH-GOGAT1 knockout mutant showed an NH_4^+-responsible inhibition of the main root elongation, when seedlings were hydroponically grown for 6 days in the presence of NH_4Cl in the culture solution (Fig. 3). The inhibition was apparently enhanced with increasing concentrations of NH_4Cl. The inhibition of root elongation was not observed when the seedlings were treated with 1 mM NO_3^-. Different sources of nitrogen showed no effect on the shoot-growth of the mutant. In normal rice roots at this stage, NADH-GOGAT1 protein transiently accumulates in the two cell-layers of root surface, epidermis and exodermis cells, following the supply of NH_4^+ (Ishiyama et al. 1998).

Metabolite profiling of both roots and shoots in rice mutants lacking *OsGS1;1* showed that there is a big increase in sugars, whereas amino acids and organic acids decreased (Kusano et al. unpublished results). Thus, in case of the *OsNADH-GOGAT1* knockout mutant, metabolic disorder is expected. The content of amino

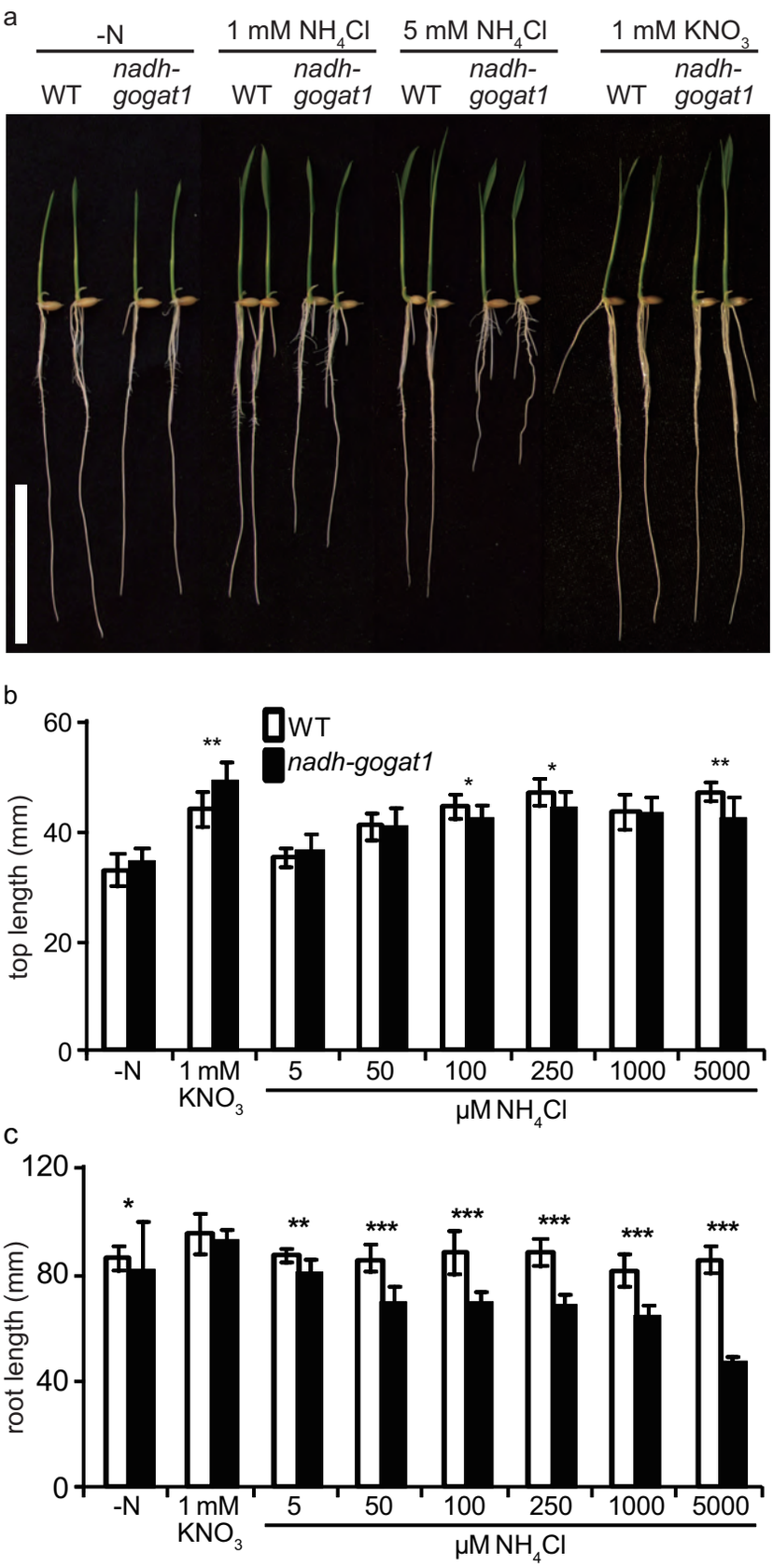

Figure.3 Phenotypic characteristics of *OsNADH-GOGAT1*-knockout mutants at seedling stage. (a) Phenotype of the knockout mutant line NG0088 (*nadh-gogat1*) grown for six days in the absence of nitrogen (-N), 1 or 5 mM NH_4Cl, or 1 mM KNO_3 after germination. Scale bar is 5 cm. (b) Comparison of top length and (c) root length of wild type (white column) and the mutant (black column). Bars are means of independent 10 samples and standard deviation values (n=10) are indicated. Significant differences between WT and nadh-gogat1 by student's t-test are indicated with asterisks: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

acids and NH_4^+ ion in roots of the mutants were determined as described previously (Tabuchi et al. 2005). As shown in Fig. 4, content of Glu (product of NADH-GOGAT1 reaction) as well as that of aspartate, asparagine and alanine were significantly reduced in the roots of the mutant, when grown in the presence of either 1mM or 5 mM NH_4^+. On the other hand, there was no significant change in Gln (substrate of the reaction) content and in other amino acids, such as leucine and valine between the roots of the mutant and the wild-type. Increase in the content of NH_4^+ ion in the mutant roots suggests that the lack of NADH-GOGAT1 causes a reduced supply of Glu to the GS1 reaction. Thus, metabolic disorder in amino acids occurs in the roots of the *OsNADH-GOGAT1* knockout mutant. In the top parts, profiles of the contents of amino acids and NH_4^+ ion were basically similar to those detected in roots, but the rate of reduction of aspartate as well as the rate of increase in NH_4^+ ion were rather gentle in the top of the knockout mutants (Fig. 5).

We surmised that the observed inhibition of the root elongation of the OsNADH-GOGAT1 knockout mutant in response to NH_4^+ may be related to the changes in endogenous contents of plant growth substances (hormones) because plant hormones play an important role as signaling molecule in the regulation of almost all phases of plant development (Kojima et al. 2009). Therefore, profiling of a number of hormones and their derivatives were determined as described by Kojima et al. (2009) using roots of the mutant and wild-type grown with 5 mM NH_4^+ for 6 days. With this sensitive and high-throughput analysis, 43 molecular species of cytokinins, auxins, abscisic acid and gibberellins can be quantitated with less than 100 mg fresh roots. Significant changes between the roots of mutant and wild-type were only detected in the content of indole-3-acetic acid (IAA: active form) and indole-3-acetyl-L-aspartate (IAAsp: inactive form of IAA) (Fig. 6). Contents of other plant

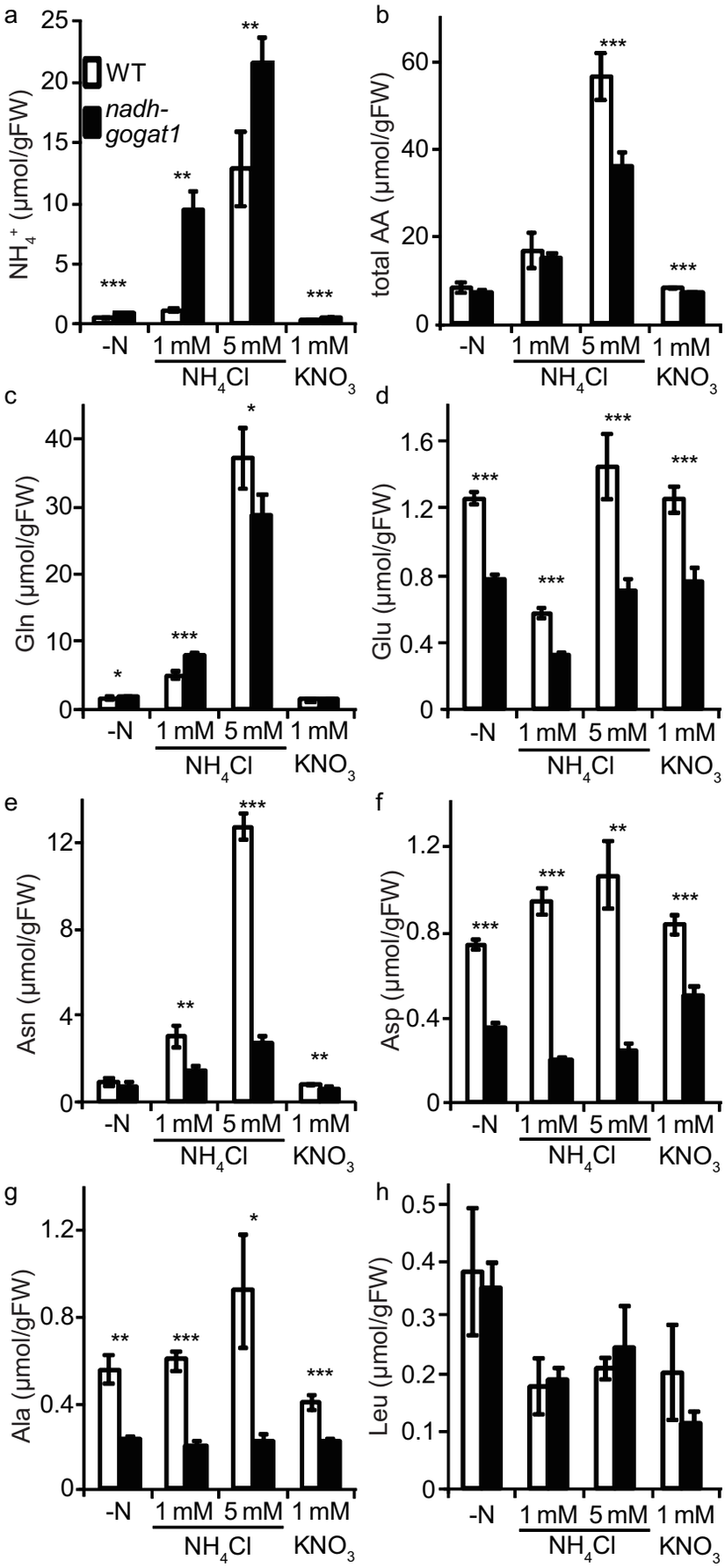

Figure.4 Comparison of free amino acids and NH_4^+ in roots of wild type (WT: white column) with those of *OsNADH-GOGAT1* knockout mutant (nadh-gogat1: black column). Contents of NH_4^+ (a), total free amino acids (b), Gln (c), Glu (d), Asn (e), Asp (f), Ala (g) and Leu (h) are indicated as µmol g-1 flesh weight of root grown as described in Fig. 2. Contents of free amino acids and NH_4^+ were determined as described by Tabuchi et al. (2005) with minor modification, in which AccQ Taq amino acid analysis column for separation module was used. Means of independent four or five samples and standard deviation values (n=4-5) are indicated. Significant differences between WT and nadh-gogat1 by student's t-test are indicated with asterisks: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

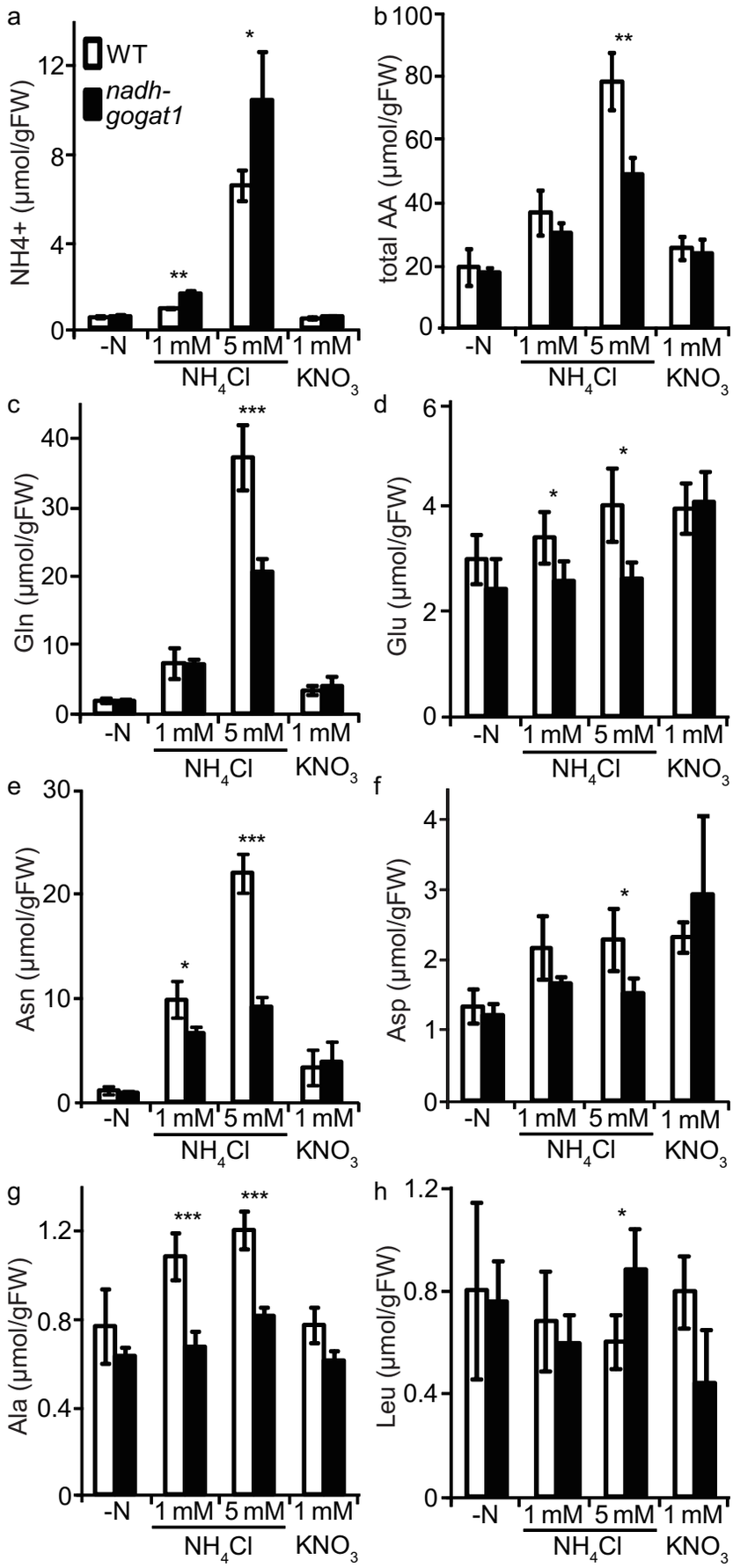

Figure. 5 Comparison of free amino acids and NH_4^+ in top part of wildtype (WT: white column) with those of *OsNADH-GOGAT1* knockout mutant (*nadh-gogat1*: black column). Plants growth conditions as well as analytical methods was the same as described in legend of Fig. 4

hormones and derivatives in the mutant roots were identical to those found in the wild type roots, as in the case of cis-zeatin (Fig. 6b). IAA content in the mutant roots was slightly higher than the wild-type roots, whereas IAAsp content was greatly decreased in the mutants.

OsNADH-GOGAT1 knockout mutation caused decrease in yield of rice

In the developing sink organs, such as young leaf blade and developing grains, the transported Gln through phloem is re-utilized for many biosynthetic processes and we have proposed that NADH-GOGAT1 is important in the reutilization of Gln (Tabuchi et al. 2007). This is mostly based on the localization studies, in which NADH-GOGAT protein was found to accumulate in vascular parenchyma cells and mestome sheath cells of developing young leaves and dorsal vascular cells of developing grains (Hayakawa et al., 1994). Expression of *OsNADH-GOGAT2* gene was not detected in developing young leaf blades and spikelet during the ripening (Tabuchi et al. 2007). Therefore, the immunologically detected NADH-GOGAT protein in the previous studies could be the NADH-GOGAT1.

In order to obtain more conclusive evidence to support our hypothesis, the *OsNADH-GOGAT1* knockout mutants were grown in paddy field until the harvest as described by Obara et al. (2004). Phenotypic characteristics of the mutants were compared with those of the wild-type and no-insertion null mutant of rice. The mutants showed slight but significant reduction in plant height, plant biomass and panicle weight (Fig. 7). Yield of rice is defined by the product of panicle number, spikelet number, proportion of well ripened grains, and spikelet weight (Sakamoto and Matsuoka 2008). When the yield components were determined in the wild-type and the mutants (Fig. 8), panicle number (Fig. 8c) and spikelet number (Fig. 8b) per plants were significantly reduced in the mutants, respectively, and

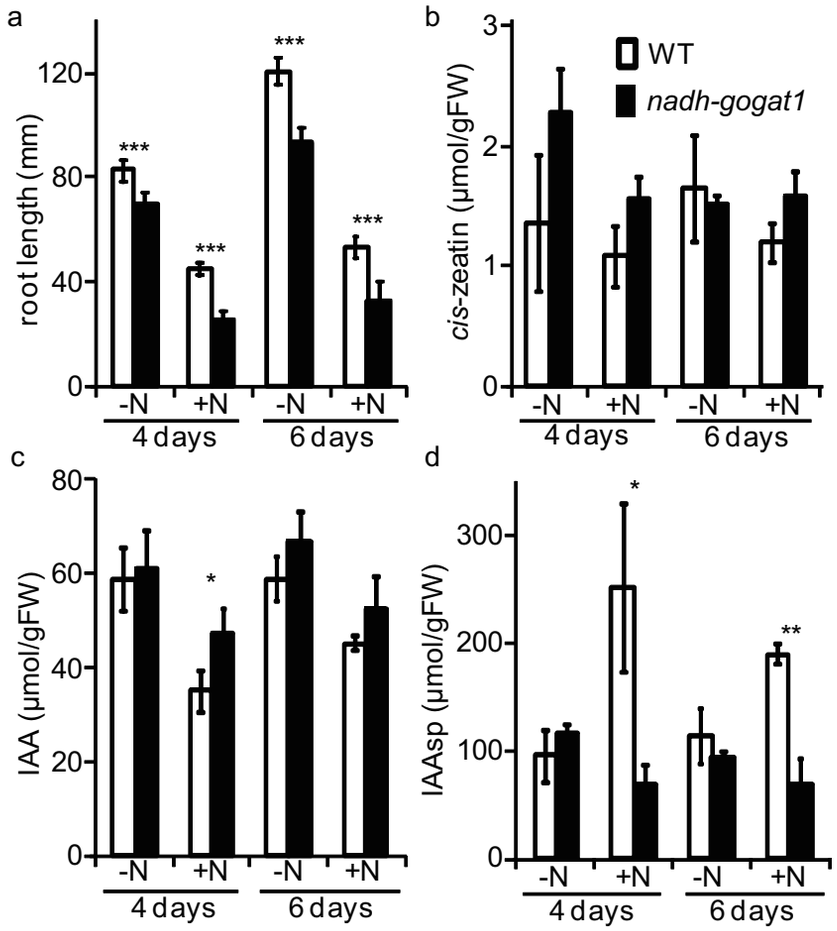

Figure. 6 Comparison of plant hormones in roots of wild-type (WT: white column) with those of *OsNADH-GOGAT1* knockout mutant (*nadh-gogat1*: black column). WT and *nadh-gogat1* were grown hydroponically in the absence of nitrogen (–N) or 5 mM NH_4Cl (+N) for 4 or 6 days after germination. a Root length. b cis-zeatin, c indole 3-acetic acid (IAA), and d indole 3-acetyl-L-aspartate (IAAsp) contents were determined as described by Kojima et al. (2009). Means of independent ten samples and standard deviation (SD) values (n = 10) for a, and those of three samples and SD values (n = 3) for b, c, and d are presented, respectively. Significant differences between WT and nadh-gogat1 by Student's t test are indicated with asterisks: **P*<0.05, ***P*<0.01, ****P*<0.001

this caused decrease in the total biomass and panicle production. On the other hand, 1,000-spikelet weight was identical to either the wild-type or no insertion null mutant (Fig. 8d) and changes in relative value of fully ripened grains was very small (Fig. 8f). The reduction of panicle number per plant is likely the cause for low productivity of the mutant, since other parameters were similar between the mutants and wild-type/null mutants. It is noted here that several T0 transgenic lines overexpressing *OsNADH-GOGAT1* gene in the indica cultivar, Kathalath, were found to have increased panicle weight per main stem (Yamaya et al. 2002). Thus, it is reasonable to suggest that the NADH-GOGAT1 is indeed important in the reutilization of Gln in developing organs.

Discussion

Assimilation of NH_4^+ and utilization within rice plants is a complex process, but is tightly related to biomass production and grain yield. Biochemical and molecular studies have led to a better understanding of the metabolic pathway of NH_4^+ assimilation (Ireland and Lea 1999; Yamaya and Oaks 2004). Genome sequencing of rice was completed in 2005 by the International Rice Genome Sequencing Project and it became apparent that there are three genes for GS1 and two genes for NADH-GOGAT in rice (Tabuchi et al. 2007). However, the precise function of each gene product in the nitrogen utilization processes in rice has largely remained unknown. The occurrence of gene families makes it difficult to characterize the physiological function of each gene product, since isoenzymes possess distinct and/or redundant functions. Reverse genetic approaches have shown that GS2 (Wallsgrove et al. 1987) and Fd-GOGAT (Kendall et al. 1986) function in the re-assimilation of NH4+ derived from the photorespiratory conversion of glycine to serine in barley. Also, GS1;1 in rice (Tabuchi et al. 2005) and GS1-3 and GS1-4 in maize (Martin et al. 2006) have been characterized. These studies indicate that these isoenzymes are involved in the control of grain production.

In rice plants, *OsGS1;2* was expressed predominantly in roots grown in the presence of NH_4^+, whereas *OsGS1;3* was specifically expressed in spikelet throughout ripening (Tabuchi et al. 2007). Expression of *OsNADH-GOGAT1* was found predominantly in the spikelet during the early stage of ripening and in a NH_4^+-responsive manner in roots; however, *OsNADH-GOGAT2* was expressed only in the mature leaf blades and sheaves (Tabuchi et al. 2007). In the current study with a knockout mutant for *OsNADH-GOGAT1*, we demonstrate that NADH-GOGAT1 functions in the primary assimilation of NH_4^+ possibly together with GS1;2. The knockout mutant showed an increase in NH_4^+ availability which led to a downregulation in the metabolism of Glu, Asp, Asn, and Ala in the roots, when the seedlings were grown hydroponically (Fig. 4). These metabolic disorders in amino acid metabolism were also observed in shoots of the mutants,although lesser to an extent than the roots. A knock-out T-DNA insertion mutant for NADH-GOGAT (*glt1-T*) of *Arabidopsis thaliana* was also isolated, showing that the mutant has a specific defect in growth and glutamate biosynthesis in leaves under non-photorespiratory conditions (Lancien et al. 2002). Although the phenotypic characteristics were hidden under normal

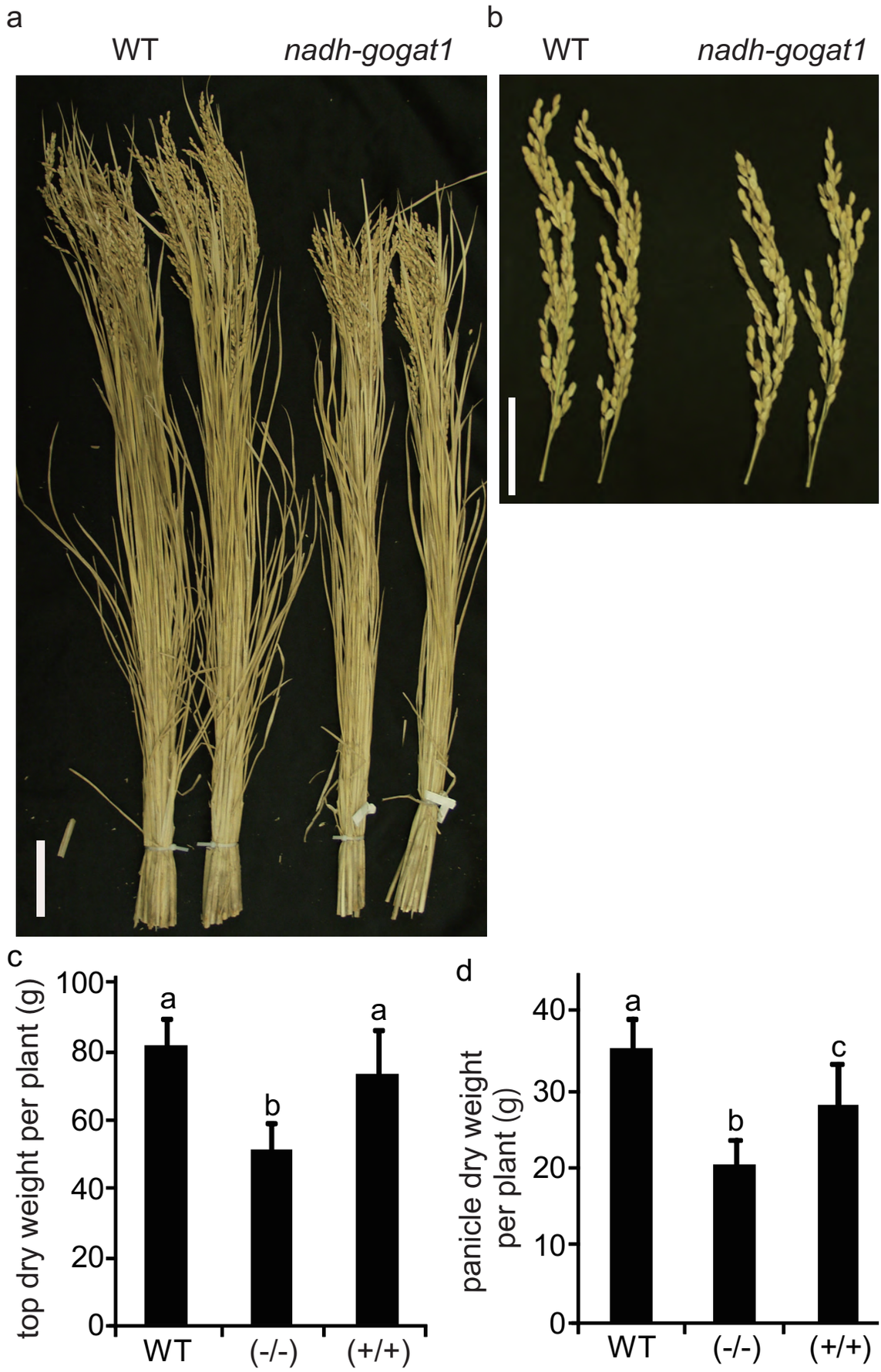

Figure. 7 Phenotypic characteristics of *OsNADH-GOGAT1*-knockout mutants (*nadh-gogat1*) at ripening stage cultivated in paddy field. a Phenotype at harvest. b Phenotype of panicle on a main stem. c Total dry weight of top part of wild-type (WT), *OsNADH-GOGAT1*- knockout mutant (-/-) and no insersion line (+/+), and d Total dry weight of panicles of wild-type (WT), *OsNADH-GOGAT1*-knockout mutant (-/-) and no-insertion line (+/+). Scale bars in a and b were 10 and 5 cm, respectively. Means of independent ten samples and standard deviation values (n = 10) are indicated in c and d. Significant differences in these three lines by one-way ANOVA are indicated by lower-case alphabet (a, b and c) on error bars

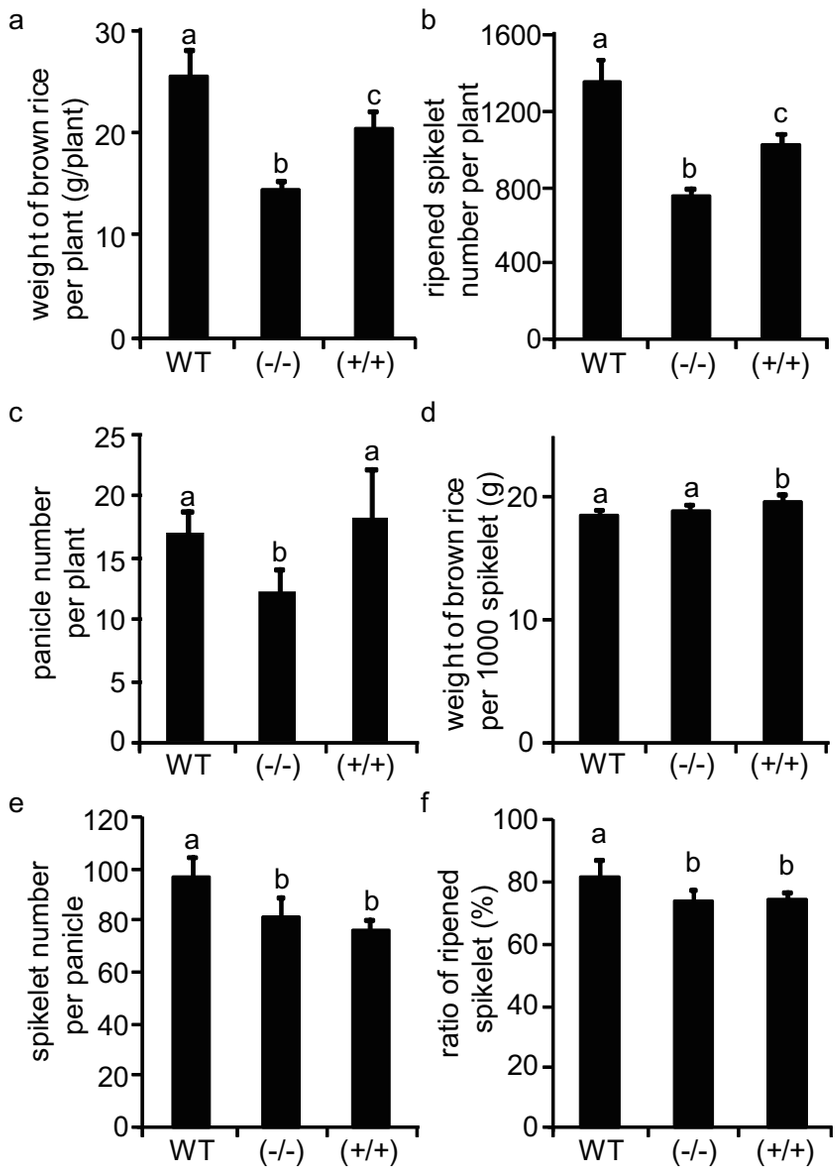

Figure. 8 Productivity of *OsNADH-GOGAT1*-knockout mutants (*nadh-gogat1*) cultivated in paddy field. a Total weight of brown rice, b total ripened spikelet number, c total panicle number, d 1,000 brown rice weight, e spikelet number per panicle and f proportion of well ripened grains of wild-type (WT), *OsNADH-GOGAT1*-knockout mutant (-/-) and no-insertion line (+/+) are indicated as means of independent five (a, b, d, e, f) or 10 (c) samples with standard deviation values (n = 5 or 10). Ripened spikelets in d were selected using NaCl solution (d = 1.06). Significant differences in those three lines by one-way ANOVA are indicated by lower-case alphabet (a, b and c) on error bars

air conditions in case of *Arabidopsis thaliana* leaves, NADH-GOGAT could function in non-photorespiratory ammonium assimilation during plant development. While the growth of *glt1-T* as compared with that of the background line only in MS medium containing 20 mM ammonium and 40 mM nitrate as nitrogen, the growth of *OsNADH-GOGAT* was compared with that of background line in various concentrations of ammonium as sole nitrogen. As we analyzed root tissues under NH_4^+ fed conditions, the importance of NADH-GOGAT1 in the primary assimilation of NH_4^+ can be clearly seen under normal growth conditions. The *OsNADH-GOGAT* knockout mutant also showed an NH_4^+-responsible inhibition of main-root elongation at the seedling stage. Since Cao et al., 1993 discovered the link between ammonium inhibition of *Arabidopsis* root growth and the phytohormone auxin, we assumed that this phenotype could be related to changes in endogenous hormone content. Thus, we profiled 43 molecular species of plant hormones and their derivatives. Both 4 and 6 day old wild type plants showed low IAA concentration under ammonium sufficient conditions, although the cis-zeatin concentration did not exhibit large differences. This observation is in good agreement with previous literature (Tian et al., 2008) suggesting a strong correlation between the inhibition of maize root growth by high nitrate supply with reduced IAA levels in roots. Contrary, IAAsp, non-active molecular forms of IAA with a conjugated amino acid, accumulated in roots of wild type plants under ammonium sufficient conditions. When IAA and IAAsp levels were compared, significant increase of IAA and decreases of IAAsp were detected only in the mutants. As exogenous IAA supply promotes primary root elongation only under nitrate sufficient conditions in maze roots (Tian et al., 2008), we expected lower IAA concentrations in *OsNADH-GOGAT1*; however this was not the case in our experiments. We posit two possible explanations for this phenomenon; first, plants' response to excess nitrogen is species specific and/or compound specific; second,IAA biosynthetic capacity or transport into the roots might be reduced resulting in dicreased of IAA content and reducing root growth rates in the mutant.

NADH-GOGAT1 could also be important in the productivity of rice grains through the development of panicle number and ripened-spikelet number on a whole plant basis. When knockout mutants were grown in paddy field (Fig. 8, their physiological characteristecs were similar to our previous studies of the over-expression of *OsNADH-GOGAT1* gene originated from japonica rice in an indica cultivar, Kathalath, where several transgenic Kathalath lines over-producing NADH-GOGAT1 under the control of their own promoter showed an increase in panicle weight on the main stem (Yamaya et al. 2002). At the same time, several lines with co-suppression of the *OsNADH-GOGAT1* gene were obtained and these co-suppressed lines showed severe reductions in main stem panicle mass . These results strongly suggest that

NADH-GOGAT1 located in vascular tissue of developing grains is indeed a key step for re-utilization of Gln remobilized from senescing organs, and thus yield. As well as Gln, Asn is a major form of nitrogen in xylem (Fukumorita and Chino 1982) and phloem sap (Hayashi and Chino 1990) in rice plants. A big decrease in the Asn contents in both the roots and shoots of the mutant seedling lacking *OsNADH-GOGAT1* gene is probably related to the decrease in yield. Importance of Asn and Gln in determining nitrogen use efficiency in relation to yield has also been discussed in maize (Cañas et al. 2009). The biochemical function of NADH-GOGAT2 remains unclear, since only trace amounts of NADH-GOGAT protein were detected in mature leaf blade and sheath of rice (Yamaya et al. 1992).

Metabolic and proteomic studies and bioinformatics have been essential tools for system analysis of plant function (Shinozaki and Sakakibara 2009). Profiling of metabolites as well as transcripts in knockout mutants provides valuable results to understand the global coordination of metabolism in plants. In our preliminary work on the profiling of metabolites in *OsGS1;1* mutants, a large imbalance between sugars and amino acids was observed (Kusano et al. unpublished results). This approach will also be applied to the *OsNADH-GOGAT1* knockout mutant in the future. These analyses will provide new information on the complex metabolic network in rice.

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