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To cite this article: Haibo Zhang, Chenggang Liang, Naohiro Aoki, Kiyoshi Kawai, Ken-ichi Takane & Ryu Ohsugi (2016) Introduction of a fungal NADP(H)-dependent glutamate dehydrogenase (*gdhA*) improves growth, grain weight and salt resistance by enhancing the nitrogen uptake efficiency in forage rice, *Plant Production Science*, 19:2, 267-278, DOI: [10.1080/1343943X.2015.1133237](https://doi.org/10.1080/1343943X.2015.1133237)

To link to this article: <https://doi.org/10.1080/1343943X.2015.1133237>



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Published online: 09 Feb 2016.



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# Introduction of a fungal NADP(H)-dependent glutamate dehydrogenase (*gdhA*) improves growth, grain weight and salt resistance by enhancing the nitrogen uptake efficiency in forage rice

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## ABSTRACT

We report the effect of the introduction of a fungal glutamate dehydrogenase gene (*gdhA*) into forage rice (cv. Momiroman) by analyzing the transgenic rice plants in terms of growth, source function, and nitrogen contents. NADP (H)-dependent glutamate dehydrogenase activities in the *gdhA*-transgenic lines were markedly higher than those in non-transgenic control plants. Plant growth analysis at the seedling stage revealed that the leaf area and shoot and root dry weights of the high *gdhA*-expressors were higher than those of control plants under both high (high N) and low nitrogen (low N) conditions. These results suggested that the source ability was enhanced by the *gdhA* introduction. This was supported by the fact that the net photosynthesis rate at the heading stage was also higher in transgenic than in control leaves. Furthermore, under both high and low N conditions, the nitrogen contents in the shoots and roots, at seedling and grain-harvest stages, were significantly higher in high *gdhA*-expressors than in control plants, indicating that nitrogen uptake was higher in transgenic than in control plants. At the harvest stage, the high *gdhA*-expressors exhibited greater panicle and spikelet numbers per plant compared with control plants, resulting in higher grain weight, under the high N conditions. In addition, *gdhA* expression in forage rice significantly enhanced their tolerance to salt stress compared to control plants. The present study showed that the introduction of a fungal *gdhA* into forage rice could lead to higher source ability, better growth and higher grain weight by enhancing nitrogen uptake efficiency.

## ARTICLE HISTORY

Received 2 October 2015  
Revised 14 November 2015  
Accepted 25 November 2015

## KEYWORDS

NADP (H)-dependent glutamate dehydrogenase; nitrogen assimilation; *Oryza sativa* L; transgenic forage rice

Continuous growth of the global population and the subsequent increase in food demand have led to a demand for increasing agricultural production. To meet this increased demand, current agricultural practices rely heavily on nitrogenous fertilizers in the form of ammonium ( $\text{NH}_4^+$ ) or nitrate ( $\text{NO}_3^-$ ). However, crop plants can utilize only 30–50% of the applied nitrogen. The unused nitrogen is inevitably leached into the underground water system, and diffuses into the atmosphere causing severe environmental problems which might lead to global warming (Bates et al., 2008) and potentially induce health problems such as gastric cancer and methemoglobinemia in humans (Luo et al., 2006; Umar et al., 2007). Therefore, there is a need to improve the ability of crop plants to utilize nitrogenous fertilizers by promoting nitrogen uptake, assimilation, and metabolism.

The main form of nitrogen available for crop plants such as rice (*Oryza sativa* L.) in irrigated paddy fields is  $\text{NH}_4^+$ . Assimilation of  $\text{NH}_4^+$  into amino acids is performed through the cooperative activity of two enzymes,

glutamine synthetase (GS), and glutamate synthase (GOGAT), referred to together as the GS/GOGAT cycle in higher plants (Lea & Mifflin, 1974). The ATP-dependent condensation of  $\text{NH}_4^+$  with glutamate is catalyzed by GS to produce glutamine. GOGAT transfers the amide nitrogen of glutamine to 2-oxoglutarate (2-OG) to form two glutamate molecules. Since the discovery of the GS/GOGAT cycle in higher plants in the 1970s, the role of glutamate dehydrogenase (GDH; EC 1.4.1.2–4) in  $\text{NH}_4^+$  assimilation has been a matter of controversy. GDH catalyzes the reversible amination of 2-OG with  $\text{NH}_4^+$  to form glutamate in the presence of NAD(P)H as a cofactor (Wootton, 1983). There are at least two distinct enzymes for GDH: NAD(H)-GDH (EC 1.4.1.2) and NADP(H)-GDH (EC 1.4.1.4). In comparison with NAD(H)-GDH, very little has been reported on NADP(H)-GDH in plants, and its role in plant metabolism remains obscure. In higher plants, the biosynthetic function of GDH seems improbable because GDH has a low affinity for  $\text{NH}_4^+$  (Harrison et al., 2000). However, in micro-organisms such as bacteria or

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Ascomycota, both NADP(H)-GDH and GS play important roles in  $\text{NH}_4^+$  assimilation (Kinghorn & Pateman, 1973). Some reports show that the affinity of GDH for  $\text{NH}_4^+$  is considerably higher in micro-organisms than in higher plants (Mifflin & Lea, 1980; Noor & Punekar, 2005; Srivastava & Singh, 1987; Wang & Tian, 2001). Mutants of *Aspergillus nidulans* lacking GDH activity showed severe reduction in growth after the supply of a source of nitrogen, whereas inactivation of GOGAT had no effect on the growth phenotype (Kinghorn & Pateman, 1973; Macheda et al., 1999). These results suggest that both GDH and GS play essential roles in the synthesis of glutamate and glutamine, respectively, during  $\text{NH}_4^+$  assimilation in micro-organisms. The presence of NADP(H)-GDH in micro-organisms led to the hypothesis that the introduction of this gene into higher plants might enhance plants' nitrogen assimilation. Some transgenic plants have been generated using the bacterial or fungal NADP(H)-GDH gene (Ameziane et al., 2000; Kisaka & Kida, 2003), some of which had enhanced biomass and yield. Kisaka and Kida (2003) reported that in tomato overexpressing the *gdhA* gene for NADP(H)-GDH from *A. nidulans*, the level of total free amino acids in fruits was 2–3-fold higher than in controls. Abiko et al. (2010) introduced a *gdhA* gene isolated from *A. niger* in food rice (cv. Yamahoushi) and found that its overexpression resulted in elevated dry weight, nitrogen content, and grain yield in transgenic plants under high nitrogen conditions.

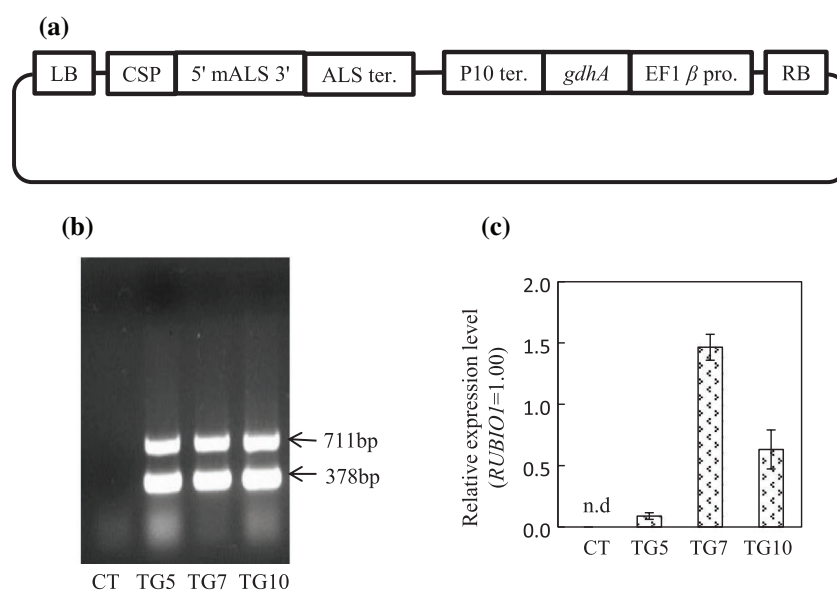
Rice is one of the most important food crops in the world and the most important grain with regard to human nutrition and caloric intake. Recently, rice has been used for feeding livestock as well as for human food. In 2011, the Japanese proportion of self-sufficiency in forage crops was only 26% of the total digestible nutrients consumed (MAFF, 2013). The Japanese government has been encouraging cultivation of feed rice, which will then be available for use in livestock diets (Sittiya et al., 2011). In Japan, forage rice, a new variety for whole-crop silage has been developed. This has high biomass production and high lodging resistance. Therefore, forage rice could be cultivated with a higher nitrogen loading than used for common rice cultivation. However, this may further increase environmental pollution due to leaching of nitrogen unused as mentioned above, as well as through emission of nitrogen oxide into air (Vitousek et al., 1997). Decreasing nitrogen fertilizer inputs by improving feed crop nitrogen use efficiency will be essential for future sustainable agriculture. Of feed rice, cv. Momiroman seems to be most suitable cultivar due to its high grain yield and its lodging resistance is superior, being evaluated as 'very strong.' According to yield trial tests by the National Institute of Crop Science, its yield of rough brown rice was 35–40% more than that of cv. Nipponbare and 8–15% more than the high yielding cv. Takanari. Total digestible nutrient yield per unit of

whole plant of Momiroman at the yellowing–ripe stage was about 8% more than that of Nipponbare. Therefore, Momiroman is a potential superior cultivar for forage rice and whole-crop silage (Hirabayashi et al., 2010). In the present study, we examined the effects of the introduction of a fungal GDH gene into Momiroman by analyzing the transgenic rice plants in terms of growth, source function, and nitrogen contents. In addition, since it has been reported that transgenic plants expressing fungal or bacterial GDH showed improved tolerance to abiotic stresses (Du et al., 2014; Lightfoot et al., 2007), we examined the effect of exogenously introduced NADP(H)-GDH on salt tolerance capacity of the control and transgenic plants at the three-leaf stage using 50, 100, and 150 mM sodium chloride (NaCl) solutions. The present study demonstrated that the introduction of a fungal GDH gene into forage rice led to higher source ability, better growth, and higher grain weight, by enhancing nitrogen uptake efficiency and salt tolerance.

## Materials and methods

### Plant materials and growth

The full-length cDNA for NADP(H)-dependent GDH was isolated from *A. niger* by reverse transcription-PCR (RT-PCR) (Abiko et al., 2010). Information on the complete nucleotide sequences and amino acid sequences for *A. niger gdhA* was obtained from public databases (GenBank Accession Number Y15784). To express introduced *gdhA* constitutively within transgenic rice plants, we used a rice elongation factor-1 beta promoter (EF1 $\beta$ ; Gene Locus Os04g0118400) and a rice prolamin 10 terminator (P10; Os03g0766000). PCR-amplified cDNA for *gdhA*, DNA for the EF1 $\beta$  promoter, and DNA for the P10 terminator were sub-cloned appropriately into a plasmid vector, pUC19 (Takara Bio, Shiga, Japan). The resultant EF1 $\beta$  promoter-*gdhA*-P10 terminator was amplified by PCR and introduced into pSTARA R-4 (Inplanta Innovations, Yokohama, Japan) with substitution of promoter and terminator region of mutated acetolactate synthase (mALS) gene to produce a binary vector for rice transformation. The resultant construct (Figure 1(a)) contained the mALS gene, regulated by callus specific promoter (CSP;Os10g0207500), which confers resistance to bispyribac-sodium (BS), an ALS-inhibiting herbicide, and thus can be used as a selectable marker for rice transformation (Kawai et al., 2007). The constructed plasmid was introduced into forage rice cv. Momiroman by *Agrobacterium*-mediated transformation (Toki et al., 2006). The BS-resistant, regenerated rice plants were grown in a glasshouse.  $T_1$  seeds were obtained from more than 10 independent lines. Three transgenic lines (TG5, TG7 and TG10) were selected for further analyses, based on the



**Figure 1.** Generation of transgenic rice plants expressing the *gdhA* gene. (a) Plasmid construct for the transformation. LB, left border; RB, right border; CSP, callus specific promoter; mALS, mutated acetolactate synthase gene; ALS ter., ALS terminator; EF1 $\beta$  pro, elongation factor 1beta promoter; P10ter, prolamin 10 terminator. (b) Genomic PCR analysis of control (CT) and transgenic (TG5, TG7 and TG10) lines. The *gdhA* gene, 711 bp; mALS gene, 378 bp. (c) Quantitative RT-PCR was analysis of *gdhA* transcripts in the leaves. RT-PCR was performed with RNA from the leaves of the T3 generations of CT, TG5, TG7, and TG10 lines. Values are expressed as the means  $\pm$  SD of eight replicates, after normalization with internal control gene (*RUBI1Q1*). The letters 'n.d.' indicates 'not detected'.

results of genomic PCR and quantitative RT-PCR analysis (Figure 1(b) and (c)) and the abundance of seeds produced.

Seeds of transgenic ( $T_2$ ; all the plants used for analyses were checked by a genomic PCR with regard to the introduction of *gdhA*) and control rice plants were sown on 10 May, and grown hydroponically in 20-liter containers with Yoshida nutrient solution in a greenhouse (day/night cycle of 14/10 h and 25/20 °C). For high nitrogen (high N) treatment, the Yoshida nutrient solution ingredients were diluted to 1/4 strength, resulting in a final concentration of 360  $\mu$ M nitrogen (180  $\mu$ M  $\text{NH}_4\text{NO}_3$ ). For low nitrogen (low N) treatment, the Yoshida nutrient solution ingredients were also diluted to 1/4 strength with the exception of nitrogen, which was further reduced to 1/16, resulting in a final concentration of 90  $\mu$ M nitrogen. To enable seedlings to gradually adapt to the change of nitrogen, the seeds were initially sown in water; after 6 d, the water was changed to 1/16 Yoshida nutrient solution for 3 d; then, the concentration of Yoshida solution was raised to 1/4 for 6 d; after that, the Yoshida solution was changed back to water for 3 d. Then, 18-d-old seedlings (3-leaf stage) were transferred to a corresponding diluted Yoshida nutrient solution (high or low N conditions). The nutrient solution was daily adjusted to pH 5.0, and the solution was exchanged every 3 d. Plants were sampled after 10 and 24 d of nitrogen supplementation. The samples for RT-PCR were divided into leaf blades, stems, and roots and immediately frozen in liquid nitrogen and kept at  $-80^\circ\text{C}$  until use. The samples

for biomass analysis were divided into leaf blades, stems, and roots.

In addition, we examined the effect of exogenously introduced NADP(H)-GDH on salt tolerance capacity of control and transgenic plants at the three-leaf stage. The plants were subjected to salt stress by, respectively, adding 50, 100, and 150 mM NaCl to 1/4 Yoshida nutrient solution for 5 d. The NADP(H)-GDH activities, dry weight, nitrogen content, and nitrogen concentration were measured.

### Genomic PCR and RT-PCR analyses of transgenic rice plants

Genomic DNA was extracted from the leaves of control and transgenic lines by a modified cetyltriethyl ammonium bromide method (Murray & Thompson, 1980). Amplification by PCR was carried out using two pairs of primers, *gdhA*-specific primers: 5'-TCCATCCTCAAGTTCCTTGG-3' and 5'-CGGAAACTTCGTCTGGGTA-3'; CSP- and mALS-specific primers: 5'-ATCGGCATCGAGACCTATCC-3' and 5'-TGTCGCTGGTGTCTTACG-3', with 35 cycles of incubation at 94 °C for 30 s, 58 °C for 90 s and 72 °C for 90 s, with a final extension at 72 °C for 5 min.

Total RNA was extracted from the fully expanded, upper-most leaves of 6-week-old seedlings (6–8 leaf stage). Total RNA was purified by RNease Plant Mini kit (Qiagen, Valencia, CA, USA), according to the

manufacturer's instructions. After elimination of DNA with TURBO DNase (Invitrogen, Carlsbad, CA, USA), total RNA was quantified by spectrophotometric analysis. First strand cDNA was synthesized from the total RNA samples (200 ng) using SuperScript III reverse-transcriptase (Life Technologies Japan, Tokyo, Japan), and then an aliquot of the first-strand cDNA mixture was used as the template for real-time quantitative PCR analysis. Each PCR (10  $\mu$ L of total volume) was performed with 1  $\mu$ L of cDNA mixtures, 5  $\mu$ L of Platinum SYBR Green qPCR SuperMix-UDG with ROX (Invitrogen, Foster, CA, USA), and *gdhA* specific primer combination; 5'-TCAAGAATGCTCTCACTGGC-3' as the forward and 5'-TGAAGGAAACACAGAAGCGA-3' as the reverse primer. The conditions used for quantitative PCR with a real-time PCR machine (ABI7300 model; Applied Biosystems, Foster, CA, USA) were 50 °C for 2 min, 95 °C for 2 min, and 40 cycles of 95 °C for 15 s and 60 °C for 31 s. The amount of product was quantified using a standard curve after normalization with transcripts from an internal control gene *RUBIQ1* (Os06g0681400).

### Enzyme assays

The frozen shoots and roots of seedlings were powdered under liquid nitrogen and homogenized using an extraction buffer: 100 mM HEPES–NaOH (pH 7.5), 1 mM EDTA (pH 7.5), 1 mM PMSF, 10% glycerol (v/v), 0.2% 2-mercaptoethanol (v/v), 0.2% Triton X-100 (v/v) and 0.2% PVPP. The homogenized tissues were centrifuged at 15,000 rpm for 20 min at 4 °C. The supernatants were then desalted using Sephadex G-25 (GE Healthcare, Buckinghamshire, UK) and NADP(H)–GDH enzyme activities were measured by the methods described by Abiko et al. (2010). The aminating activity of NADP(H)–GDH was determined in a reaction mixture (1.0 mL of final volume) containing 100 mM Tris–HCl, 10 mM  $\text{NH}_4\text{Cl}$ , 10 mM 2-OG, and 0.1 mM  $\beta$ -NADPH at pH 8.0. The deaminating activity of NADP(H)–GDH was determined in a reaction mixture (1.0 mL final volume) containing 100 mM Tris–HCl, 100 mM L-glutamate, and 0.4 mM  $\beta$ -NADP<sup>+</sup> at pH 9.3. The reaction was routinely started by the addition of coenzyme ( $\beta$ -NADPH or  $\beta$ -NADP<sup>+</sup>, respectively). The NADP(H)–GDH activities were measured by monitoring the change in absorbance at 340 nm with a spectrophotometer (DU800, Beckman Coulter, Fullerton, CA, USA). Soluble protein was measured by Bradford protein assay (Bradford, 1976), using a Protein Assay Kit (Bio-Rad Laboratories, Hercules, CA, USA) and bovine serum albumin as a standard.

### Measurement of dry weight, nitrogen, and carbon contents

Leaf blades, stems, and roots of 10- and 24-d-old seedlings were harvested and dried at 80 °C for 3 d. Dried samples were weighed and ground with Multi-Beads Shocker (Yasui-Kikai, Osaka, Japan). The nitrogen and carbon contents of samples were measured using an NC analyzer (vario MAX CN model; Elementar, Hanau, Germany) according to the manufacturer's instructions.

In addition, to evaluate nitrogen uptake efficiency at the seedling stage, two-week-old control and three different transgenic plants were treated for 4 h under high and low N conditions, and then nitrogen contents remained in the culture solution were determined.

### Measurement of photosynthetic rates

Photosynthetic rate ( $P_n$ ) was measured in the attached upper-most, fully expanded leaves using a CIRAS-3 Portable Photosynthesis System (PP Systems, MA, USA). All measurements were performed at 28 °C and 75% relative humidity, with the cuvette flow constant at 300 mL  $\text{min}^{-1}$ . To obtain a light response curve, the leaf chamber  $\text{CO}_2$  concentration was maintained at 390  $\mu\text{mol mol}^{-1}$ , and  $P_n$  was measured at the following photosynthetic photon flux densities: 2000, 1,500, 1,000, 500, 300, 200, 100, 50, 20, and 0  $\mu\text{mol m}^{-2} \text{s}^{-1}$ .

### Statistical analysis

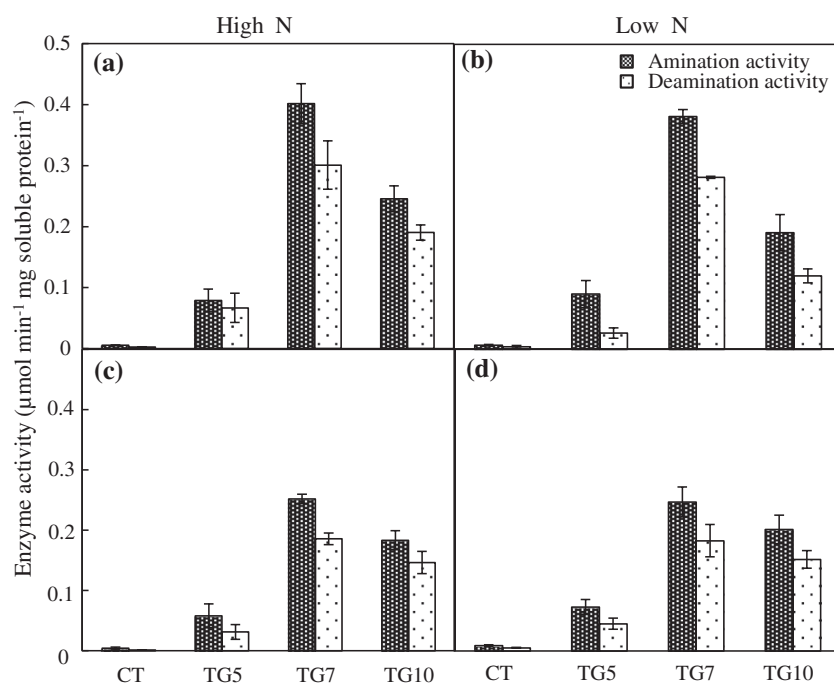
Data were analyzed using one-way ANOVA (SPSS 13.0 for Windows7; SPSS, Chicago, IL, USA). Significant differences were determined based on  $p < 0.05$ .

## Results

### Expression level and GDH activities of transgenic rice plants expressing *gdhA*

Transgenic lines expressing *gdhA* under the control of the EF1 $\beta$  promoter were first confirmed by PCR analysis using *gdhA* (amplified fragment 711 bp) and *ALS* (the selection marker, amplified fragment 378 bp) specific primers and further identified by semi-quantitative RT-PCR (Figure 1(b) and (c)). These results indicated that *gdhA* was successfully introduced and expressed in three independent transgenic lines (TG5, TG7, and TG10), although the expression level was lower in TG5 than in TG7 and TG10.

In addition to molecular confirmation, GDH activities were measured in the shoot and root of control and transgenic rice plants. The GDH activities were markedly higher in transgenic lines than in control line in both the aminating and deaminating directions, regardless of nitrogen

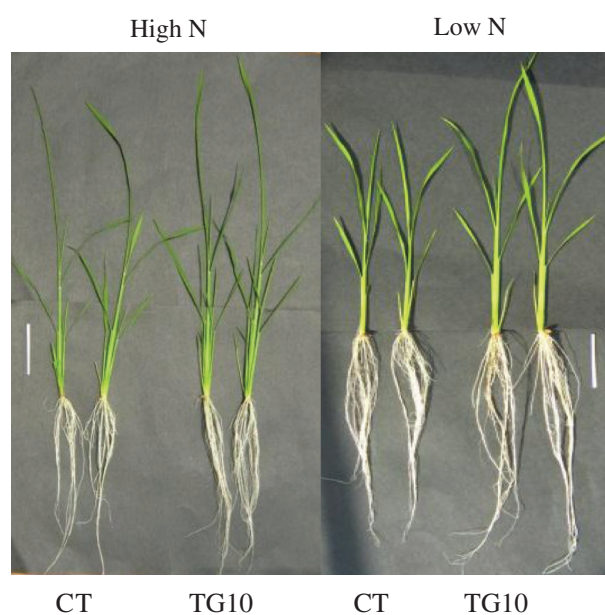


**Figure 2.** NADP(H)-GDH activities. Aminating (black bars) and deaminating (white bars) activities of NADP(H)-GDH in the shoot (a, b) and root (c, d) were measured in control (CT) and transgenic (TG5, TG7 and TG10) lines under high (a, c) and low (b, d) N conditions after 24 d treatment. Data represent the mean values  $\pm$  SD ( $n = 3$ ). Statistical analysis of the data was performed by one-way ANOVA.

conditions (Figure 2). Furthermore, the *in vitro* aminating activity of GDH was higher than its deaminating activity in the shoots and roots of transgenic lines in both high and low N conditions. The GDH activities were higher in TG7 and TG10 than in TG5 regardless of nitrogen conditions (Figure 2). We called the former two ‘high *gdhA*-expressors’ and the latter ‘low *gdhA*-expressors.’

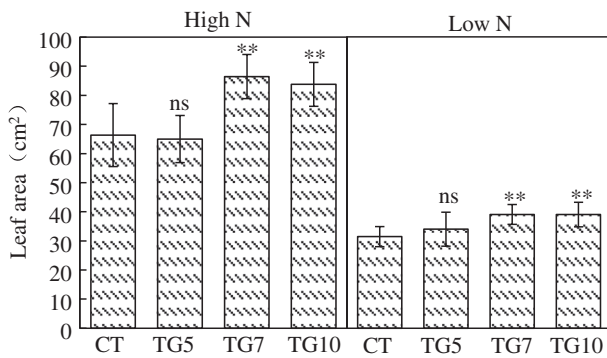
#### **Effect of *gdhA* expression on growth, nitrogen content, and nitrogen uptake efficiency at the seedling stage**

We examined the growth of the control and transgenic lines under high and low N conditions. The control plants grew normally with bright green leaves in Yoshida nutrient solutions containing 360  $\mu$ M nitrogen, indicating that there was sufficient nitrogen and other nutrients for the seedlings. However, for 90  $\mu$ M nitrogen, control plants exhibited elongated roots and light-green leaves, suggesting that there was insufficient nitrogen for normal growth (Figure 3). Plant growth analysis at the seedling stage revealed that the leaf area and leaf, stem, and root dry weights were significantly higher for high *gdhA*-expressors than for control in both high and low N plants, while low *gdhA*-expressors showed higher dry weight only under high N conditions (Figures 4 and 5). The stem nitrogen concentrations were higher for high *gdhA*-expressors than control line under low N conditions, but

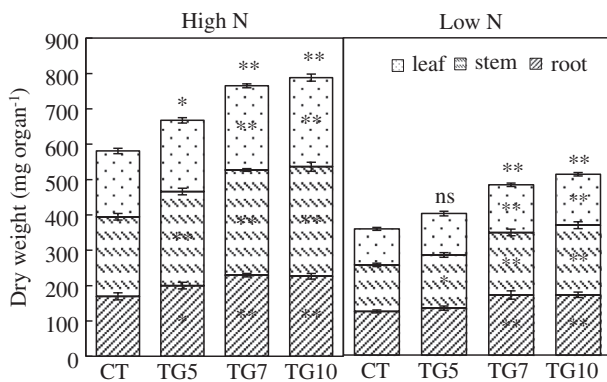


**Figure 3.** Phenotype of control (CT) and transgenic (TG10) lines under high and low N conditions after 10 d treatment. Bars = 5 cm.

were opposite under high N conditions (Figure 6). The nitrogen contents in leaves, stems, and roots were significantly higher for high *gdhA*-expressors than control line under both high and low N conditions (Figure 7). The nitrogen contents remaining in the Yoshida nutrient solution after the treatment for the three transgenic lines



**Figure 4.** Leaf area of control (CT) and transgenic (TG5, TG7 and TG10) lines under high and low N conditions at the seedling stage. Data represent the mean values  $\pm$  SD ( $n = 9$ ). Statistical analysis of the data was performed by one-way ANOVA. Asterisks indicate that the mean values of TG5, TG7, and TG10 lines are significantly different from that of CT at  $p < 0.05$  (\*) and  $p < 0.01$  (\*\*). The letters 'ns' indicate not significantly different from CT at  $p < 0.05$ .

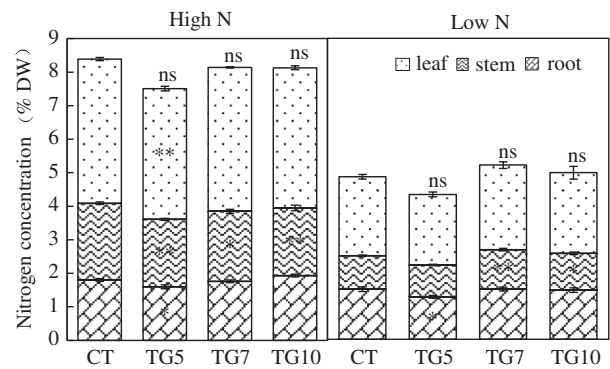


**Figure 5.** Dry weight of control (CT) and transgenic (TG5, TG7 and TG10) lines under high and low N conditions at the seedling stage. Data represent the mean values  $\pm$  SD ( $n = 9$ ). Statistical analysis of the data was performed by one-way ANOVA. Asterisks indicate that the mean values of TG5, TG7 and TG10 lines are significantly different from that of CT at  $p < 0.05$  (\*) and  $p < 0.01$  (\*\*). The letters 'ns' indicate not significantly different from CT at  $p < 0.05$ .

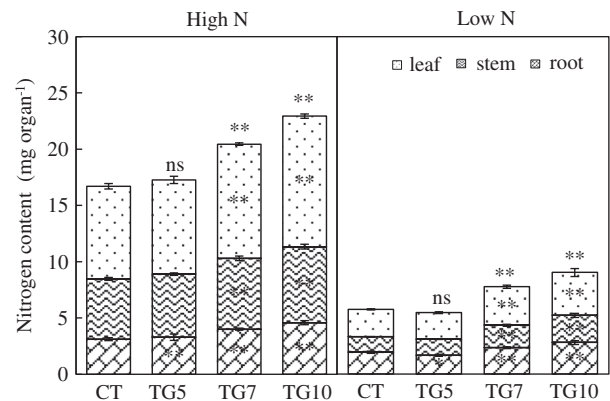
were significantly lower than that for control line under both high and low N conditions, indicating that the transgenic lines took up more nitrogen than did the control line (Figure 8).

### Effect of *gdhA* expression on tolerance of salt stress at the seedling stage

According to previous reports, transgenic plants expressing fungal or bacterial GDHs can show improved drought or herbicide tolerance (Ameziane et al., 2000; Du et al., 2014; Lightfoot et al., 2007; Nolte et al., 2004). Therefore, the introduction of NADP(H)-GDH from micro-organisms could improve plant tolerance under stress conditions. To examine the tolerance of transgenic rice plants to salt, the

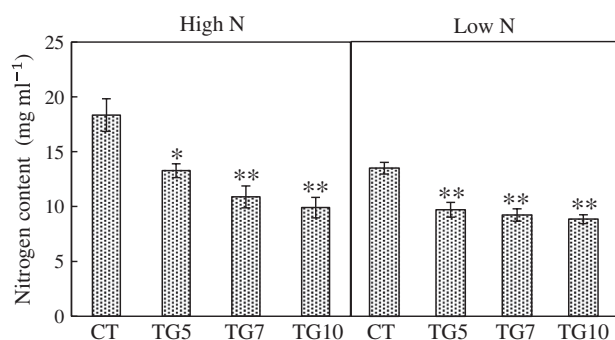


**Figure 6.** Nitrogen concentration of control (CT) and transgenic (TG5, TG7 and TG10) lines under high and low N conditions at the seedling stage. Data represent the mean values  $\pm$  SD ( $n = 4$ ). Statistical analysis of the data was performed by one-way ANOVA. Asterisks indicate that the mean values of TG5, TG7 and TG10 lines are significantly different from that of CT at  $p < 0.05$  (\*) and  $p < 0.01$  (\*\*). The letters 'ns' indicate not significantly different from CT at  $p < 0.05$ .



**Figure 7.** Nitrogen contents of control (CT) and transgenic (TG5, TG7, and TG10) lines under high and low N conditions at the seedling stage. Data represent the mean values  $\pm$  SD ( $n = 4$ ). Statistical analysis of the data was performed by one-way ANOVA. Asterisks indicate that the mean values of TG5, TG7, and TG10 lines are significantly different from that of CT at  $p < 0.05$  (\*) and  $p < 0.01$  (\*\*). The letters 'ns' indicate not significantly different from CT at  $p < 0.05$ .

seedlings of control and transgenic lines at the three-leaf stage were subjected to salt stress. The NADP(H)-GDH activities were significantly higher for transgenic than control plants under high salt conditions. Moreover, amination activity was higher than deamination activity in shoots of transgenic lines (Figure 9). The transgenic lines remained healthy with green leaves and stems, whereas leaves of control exhibited chlorosis following NaCl treatment, especially for 150 mM NaCl (Figure 10). The dry weights of high *gdhA*-expressors were significantly higher than control line in all NaCl treatments, and dry weights for low *gdhA*-expressors were also significantly higher than



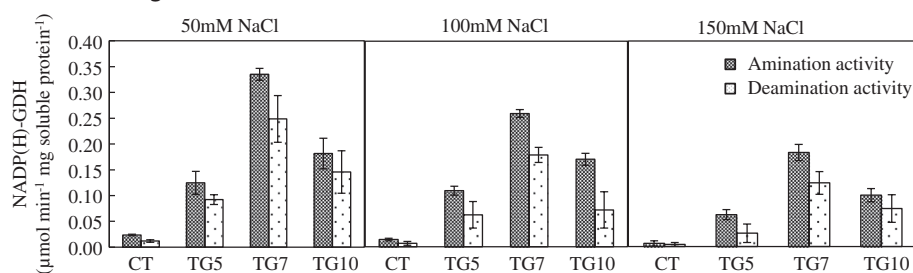
**Figure 8.** Nitrogen contents of control (CT) and transgenic (TG5, TG7, and TG10) lines remained in the culture solution after 4 h high and low N treatment at the seedling stage. Data represent the mean values  $\pm$  SE ( $n = 5$ ). Statistical analysis of the data was performed by one-way ANOVA. Asterisks indicate that the mean values of TG5, TG7 and TG10 lines are significantly different from that of CT at  $p < 0.05$  (\*) and  $p < 0.01$  (\*\*).

control line under the 150 mM NaCl treatment. Compared with control conditions, the dry weight of control line dropped to 60.3%, while the TG5, TG7, and TG10 dropped to 63.2, 73.6, and 65.9%, respectively, with 150 mM NaCl treatment (Table 1). The nitrogen concentration of low

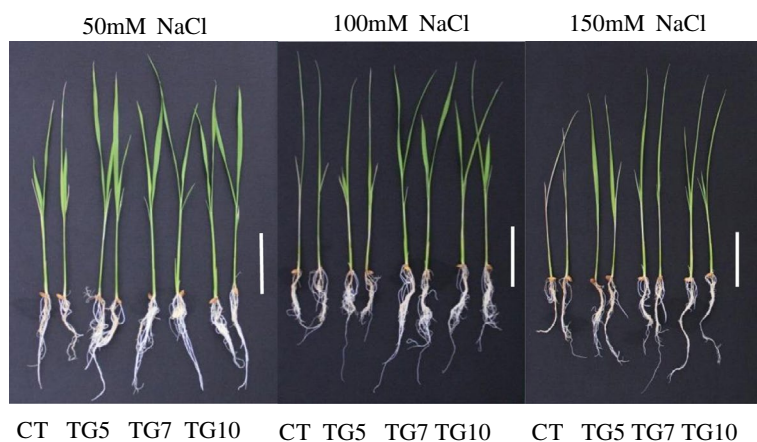
*gdhA*-expressors was higher than control line with 150 mM NaCl treatment, but there were no differences between the other lines and control regardless of NaCl treatment. The nitrogen content was significantly higher for high *gdhA*-expressors than control line in all NaCl treatments, and was also significantly higher for low *gdhA*-expressors than control line under 150 mM NaCl treatment (Figures 11 and 12).

### Effect of *gdhA* expression on the Pn at the heading stage

The increases in dry weight implied an increase in carbon assimilation, which could be mainly due to enhanced Pn. To validate this hypothesis, the light responses of Pn were measured for both transgenic and control lines. The results show that the Pn under high light intensity was higher in all transgenic leaves than in control leaves, regardless of nitrogen conditions at the heading stage (Figure 13).



**Figure 9.** Aminating (black bars) and deaminating (white bars) activities of NADP (H)-GDH in the shoot were measured in control (CT) and transgenic (TG5, TG7, and TG10) lines after 1 week treatment under different salt stress conditions. Data represent the mean values  $\pm$  SD ( $n = 3$ ). Statistical analysis of the data was performed by one-way ANOVA.



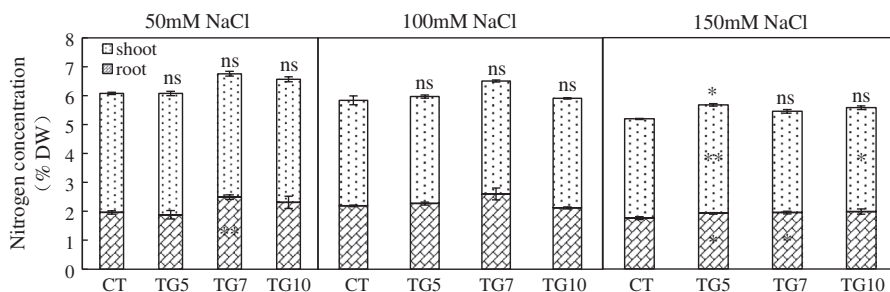
**Figure 10.** Phenotypic of control (CT) and transgenic (TG5, TG7 and TG10) lines after 1 week treatment under different salt stress conditions. Bars = 5 cm.



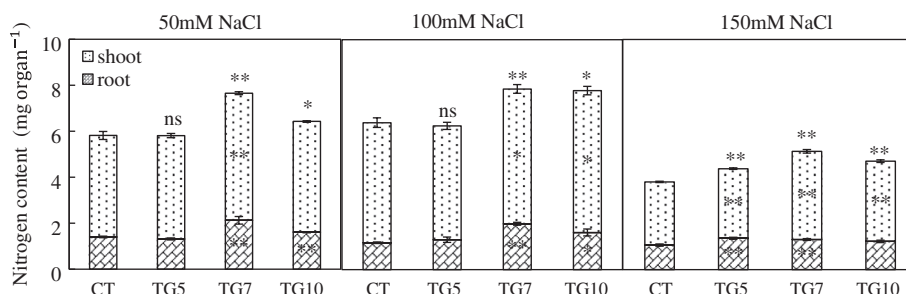
**Table 1.** Effect of different salt stress on dry weight mg of rice seedlings.

NaCl (mM)	CT	TG5	TG7	TG10
0	200.8 ± 5.2	215.9 ± 7.8	218.1 ± 7.1	216.7 ± 7.6
50	171.1 ± 1.6 (85.2)	177.8 ± 1.6 (82.3)	224.6 ± 2.3** (103.0)	203.1 ± 1.0** (93.7)
100	161.5 ± 5.4 (80.4)	166.6 ± 3.3 (77.1)	196.9 ± 3.8** (90.3)	191.2 ± 2.5** (88.2)
150	121.1 ± 3.0 (60.3)	136.6 ± 1.4** (63.2)	160.5 ± 1.1** (73.6)	142.9 ± 1.2** (65.9)

Notes. Data represent the mean values ± SD ( $n = 6$ ). Statistical analysis of the data was performed by one-way ANOVA. Asterisks indicate that the mean values of transgenic (TG5, TG7 and TG10) lines are significantly different from that of control (CT) line at  $p < 0.05$  (\*) and  $p < 0.01$  (\*\*). Data in parentheses are percentage of treated/control.



**Figure 11.** Nitrogen concentration of control (CT) and transgenic (TG5, TG7, and TG10) lines under different salt stress conditions. Data represent the mean values ± SD ( $n = 4$ ). Statistical analysis of the data was performed by one-way ANOVA. Asterisks indicate that the mean values of TG5, TG7 and TG10 lines are significantly different from that of CT at  $p < 0.05$  (\*) and  $p < 0.01$  (\*\*). The letters 'ns' indicate not significantly different from CT at  $p < 0.05$ .



**Figure 12.** Nitrogen contents of control (CT) and transgenic (TG5, TG7 and TG10) lines under different salt stress conditions. Data represent the mean values ± SD ( $n = 4$ ). Statistical analysis of the data was performed by one-way ANOVA. Asterisks indicate that the mean values of TG5, TG7, and TG10 lines are significantly different from that of CT at  $p < 0.05$  (\*) and  $p < 0.01$  (\*\*). The letters 'ns' indicate not significantly different from CT at  $p < 0.05$ .

### Effect of *gdhA* expression on nitrogen content and concentration at the harvest stage

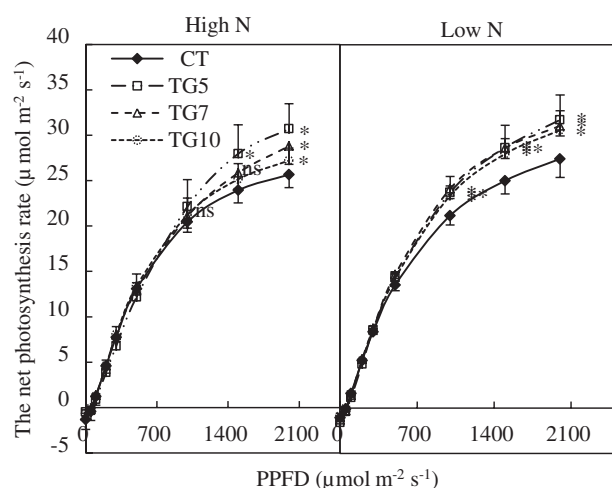
The nitrogen concentrations of transgenic lines did not differ from control line (Figure 14). In both high and low N plants, the nitrogen contents of high *gdhA*-expressors were significantly higher than those of control line, while there was only a slight increase in N content in the low *gdhA*-expressors at the harvest stage (Figure 15).

Under high N conditions, high *gdhA*-expressors exhibited higher panicle numbers and spikelet numbers per plant, resulting in higher grain weight compared with control line. In TG7, similar enhancement was also observed under low N conditions. In high *gdhA*-expressors, the

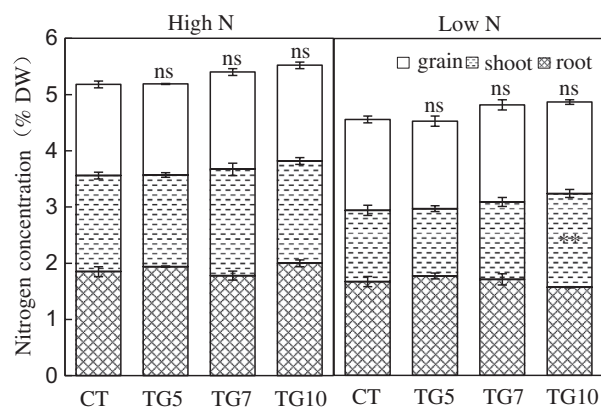
nitrogen uptake efficiencies were estimated to be higher than that in control and TG5 (Table 2).

### Discussion

GDH is a family of enzymes catalyzing reversible deamination of L-glutamate to 2-OG, directly connected to the TCA cycle (Fisher, 1985) and thereby playing a key role in providing a link between carbon and nitrogen metabolism. GDHs are ubiquitous and present in bacteria as well as in most sub-cellular compartments of the eukaryotic cell. This enzyme family has drawn much attention because assimilation of ammonium via GDH is more energy-efficient than via GOGAT (Fd-GOGAT and NADH-GOGAT) (Helling,

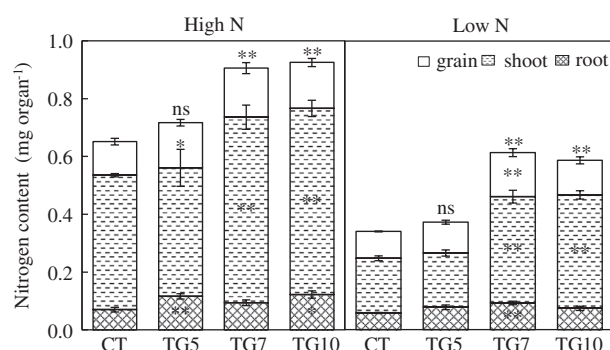


**Figure 13.** The light response curves of Pn of control (CT) and transgenic (TG5, TG7 and TG10) lines under high and low N conditions at the heading stage. Data represent the mean values  $\pm$  SD ( $n = 4$ ). Statistical analysis of the data was performed by one-way ANOVA. Asterisks indicate that the mean values of TG5, TG7, and TG10 lines are significantly different from that of CT at  $p < 0.05$  (\*) and  $p < 0.01$  (\*\*). The letters 'ns' indicate not significantly different from CT at  $p < 0.05$ .



**Figure 14.** Nitrogen concentration of control (CT) and transgenic (TG5, TG7, and TG10) lines under high and low N conditions at the harvest stage. Data represent the mean values  $\pm$  SD ( $n = 4$ ). Statistical analysis of the data was performed by one-way ANOVA. Asterisks indicate that the mean values of TG5, TG7, and TG10 lines are significantly different from that of CT at  $p < 0.05$  (\*) and  $p < 0.01$  (\*\*). The letters 'ns' indicate not significantly different from CT at  $p < 0.05$ .

1998; Windass et al., 1980). In this study, we successfully produced transgenic forage rice plants that expressed a fungal NADP(H)-GDH (*gdhA*) under the control of a constitutive EF1 $\beta$  promoter (Figure 1). Introduction of *gdhA* led to a significant increase in the NADP(H)-GDH activity in transgenic lines, with especially the high *gdhA*-expressors (TG7 and TG10) showing higher activity. The amination activities in shoots and roots of transgenic plants were considerably higher than the deamination activities under



**Figure 15.** Nitrogen contents of control (CT) and transgenic (TG5, TG7, and TG10) lines under high and low N conditions at the harvest stage. Data represent the mean values  $\pm$  SD ( $n = 4$ ). Statistical analysis of the data was performed by one-way ANOVA. Asterisks indicate that the mean values of TG5, TG7, and TG10 lines are significantly different from that of CT at  $p < 0.05$  (\*) and  $p < 0.01$  (\*\*). The letters 'ns' indicate not significantly different from CT at  $p < 0.05$ .

both nitrogen treatment conditions (Figure 2). Our results are consistent with those of several previous studies that showed high NADP(H)-GDH activities in transgenic rice or potato by the introduction of a *gdh* gene from fungi or bacteria (Abiko et al., 2010; Du et al., 2014; Egami et al., 2012; Zhou et al., 2014).

Introduction of an exogenous GDH gene into tobacco (Mungur et al., 2005) or crop plants may improve  $\text{NH}_4^+$  assimilation, raise biomass production, and enhance the absorption and utilization of nitrogen. Some transgenic crops have already been generated using a fungal NADP(H)-GDH gene, such as *NiGDH* of *Neurospora intermedia* (Wang & Tian, 2001) and *gdhA* of *A. nidulans* (Kisaka & Kida, 2003) – these transgenic plants showed high nitrogen utilization efficiency. Previously, our research group reported expression of a *gdhA* isolated from *A. niger* in transgenic food rice ('Yamahoushi') plants and that the shoot dry weight and nitrogen content were elevated only under high N conditions (500  $\mu\text{M}$ ) (Abiko et al., 2010). The present study using transgenic forage rice ('Momiroman') plants showed that the shoot dry weight and nitrogen content of high *gdhA*-expressors were significantly higher than control plants under both high (360  $\mu\text{M}$ ) and low (90  $\mu\text{M}$ ) nitrogen conditions at seedling stage (Figures 5 and 7). Although the different outcomes between the two studies may result from the rice cultivar used as host and/or the promoter used to drive the transgene, there are two possible explanations for this finding. First, that Abiko et al. (2010) used 50  $\mu\text{M}$ , but we used 90  $\mu\text{M}$  as the low N condition; and, second, they used  $\text{NH}_4^+$  as the sole nitrogen source, while we used both  $\text{NO}_3^-$  and  $\text{NH}_4^+$ . Some research has shown that partial  $\text{NO}_3^-$  nutrition can improve growth and nitrogen uptake efficiency of rice (Duan et al.,

**Table 2.** The agronomic traits and nitrogen uptake efficiency of control (CT) and transgenic lines (TG5, TG7, and TG10) under high and low N conditions.

Treatment	Line	Panicle number (plant <sup>-1</sup> )	Spikelet number (plant <sup>-1</sup> )	1,000-grain weight (g)	Grain weight (g plant <sup>-1</sup> )	Sink capacity (g plant <sup>-1</sup> )	Nitrogen uptake efficiency <sup>a</sup> (%)
High N	CT	5.0 ± 0.3	694.0 ± 42.1	24.7 ± 0.6	6.1 ± 0.9	17.2 ± 2.0	44.5 ± 0.6
	TG5	5.0 ± 0.5	723.0 ± 39.3	24.6 ± 0.5	8.8 ± 0.7*	17.8 ± 1.0	46.0 ± 5.6
	TG7	6.3 ± 0.2**	977.7 ± 42.5*	23.5 ± 0.7	8.2 ± 1.3*	22.9 ± 2.5	62.2 ± 3.7**
	TG10	6.2 ± 0.3*	1018.3 ± 48.1*	23.8 ± 0.8	8.5 ± 1.0*	24.1 ± 1.9*	61.5 ± 3.0**
Low N	CT	4.3 ± 0.2	630.2 ± 44.5	24.1 ± 0.2	6.3 ± 0.4	15.2 ± 1.2	40.3 ± 1.2
	TG5	3.8 ± 0.2	519.0 ± 36.6	25.7 ± 0.6*	6.8 ± 0.2	13.4 ± 1.1	42.0 ± 1.4
	TG7	5.3 ± 0.4	1019.3 ± 48.3**	24.4 ± 0.6	9.9 ± 0.8**	25.0 ± 1.8**	74.3 ± 2.7**
	TG10	4.5 ± 0.2	670.7 ± 42.7	25.2 ± 0.4*	6.6 ± 0.8	16.7 ± 1.7	73.1 ± 3.0**

Notes. Data represent the mean values ± SD (*n* = 6). Statistical analysis of the data was performed by one-way ANOVA.

Asterisks indicate that the mean values of

transgenic (TG5, TG7, and TG10) lines are significantly different from that of control (CT) line at *p* < 0.05 (\*) and *p* < 0.01 (\*\*).

<sup>a</sup>Nitrogen uptake efficiency = nitrogen content in plant / total amount of nitrogen supplied in Yoshida nutrient solution

2007; Herbert et al., 1999). In addition, Abiko et al. (2010) observed no significant differences in root dry weight and nitrogen content between the transgenic lines and the control line, while our results showed that the root dry weight and nitrogen content of high *gdhA*-expressors were also significantly higher than control plants under both high and low nitrogen conditions (Figures 5 and 7). Expression of *gdhA* in transgenic rice plants results in direct assimilation of NH<sub>4</sub><sup>+</sup> absorbed from the roots (Abiko et al., 2010), and NH<sub>4</sub><sup>+</sup> metabolism can enhance by the presence of NO<sub>3</sub><sup>-</sup> (Herbert et al., 1999). Data shown in Figure 8 appear to indicate that the ability for nitrogen uptake in the roots is higher in high *gdhA*-expressors than in control plants. Collectively, our results suggest that introduction of *gdhA* led to improved growth, dry weight and nitrogen content under low N conditions at the seedling stage by enhancing nitrogen uptake efficiency.

By 2009, 35% of all paddy fields in Japan had been abandoned (MAFF, 2011). A shift from rice to alternative crops such as soybean, barley, buckwheat, and colza has been promoted by the Japanese Government. However, many paddy fields are not suitable for upland crops owing to difficulty in draining excess water from the soil. Forage rice is an alternative that can adequately maintain productivity of paddy fields (Asada et al., 2013). However, paddy fields with appreciable soil salinity are a major problem for forage rice (Hammecker et al., 2012) and the salt tolerance of forage rice has not been reported. Some research has shown that the NH<sub>4</sub><sup>+</sup> concentration in plant tissues increases under salt stress (Bourgeais-Chaillou et al., 1992; Lutts et al., 1999). NADH-GDH activity was enhanced with increased NH<sub>4</sub><sup>+</sup> concentration, alleviating the toxic effect of NH<sub>4</sub><sup>+</sup> accumulation in tissues (Zhou et al., 2004). Another report showed that leaf GS and GOGAT activities were not affected by NaCl (Nguyen et al., 2005). In the present study, the NADP(H)-GDH activity decreased with increased NaCl concentrations, but the NADP(H)-GDH activities of transgenic lines were significantly higher than control line

under different NaCl treatments (Figure 9). The dry weight and nitrogen content of high *gdhA*-expressors were significantly higher than those of control line under different salt stress conditions; and low *gdhA*-expressors also showed similar enhancement under 150 mM NaCl treatment (Figure 12 and Table 1). Our data indicated that transgenic plants expressing fungal *gdhA* significantly enhanced their tolerance to salt stress compared to control line. Overall, the transgenic lines with higher tolerance to salt stress maintained higher NADP(H)-GDH activity, which played an important role in decreasing the NH<sub>4</sub><sup>+</sup> accumulation in tissues under salt stress.

Egami et al. (2012) introduced a fungal *gdhA*, encoding NADP(H)-GDH, from *A. nidulans* into potato and found that transgenic plants had enhanced photosynthetic rates, biomass production, and carbon and nitrogen contents compared with non-transgenic plants. In our study, leaf areas were markedly larger for the high *gdhA*-expressors than control line at the seedling stage (Figure 4). At the heading stage, the photosynthetic rates were higher under high light intensity for transgenic lines than for control under high and low N conditions (Figure 13). These results suggest that transgenic lines had an enhanced capability for photosynthesis. The *gdhA* transgenic tobacco plants grown in the field produced significantly higher grain weight than did control plants (Ameziane et al., 2000). Abiko et al. (2010) found that the introduction of a fungal *gdhA* gene encoding NADP(H)-dependent GDH into rice led to better growth and yields. Zhou et al. (2014) reported that grain yields of *PcGDH* transgenic plants underwent no apparent changes, yet the 1,000-grain weights and panicle numbers were augmented significantly compared with control plants. In most studies, yield data were obtained from field trials, but hydroponic experiments are a more effective way to reduce the influence of factors or substances other than that of nitrogen. The hydroponic experimental data from our study indicate that the high *gdhA*-expressors exhibited higher panicle numbers and spikelet numbers per plant,

resulting in higher grain weight compared with control line under high N conditions. In TG7, similar enhancement effects were also observed under the low N conditions. In conclusion, the results suggest that the introduction of a fungal GDH gene into forage rice plants led to higher source ability, dry matter production, and grain weight by enhancing the nitrogen uptake efficiency. Therefore, *gdhA* introduction may be useful in improving forage rice cultivars to more effectively utilize nitrogenous fertilizers, on which current agricultural practices rely heavily. It is important to note that the impact of *gdhA* introduction differs according to the expression level of the foreign gene: in TG5, the low *gdhA*-expressor, the NADP(H)-GDH activity, nitrogen content, and nitrogen uptake efficiency were considerably lower than those observed in the two high *gdhA*-expressors (TG7 and TG10), although dry matter production during seedling stage and photosynthetic capacity at the heading stage were higher than control line. Further study is required concerning the relationship between the expression level of introduced *gdhA* and its metabolic and physiological impacts, including the effect on the GS/GOGAT cycle and nitrogen utilization.

## Abbreviations

GDH, Glutamate dehydrogenase; GOGAT, Glutamate synthase; GS, Glutamine synthetase; 2-OG, 2-Oxoglutarate; RT-PCR, reverse transcription polymerase chain reaction.

## Acknowledgments

We thank Takayuki Asano and Tomohiro Kurai, The University of Tokyo, for their expertise in making the construct for rice transformation.

## Disclosure statement

No potential conflict of interest was reported by the authors.

## Funding

This work was supported by JSPS KAKENHI [grant number 23248004].

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\*In Japanese with English summary.

\*\*In Japanese.