博士論文

Physiological analyses of transgenic rice expressing a fungal glutamate dehydrogenase gene

（グルタミン酸脱水素酵素遺伝子を導入したイネの生理学的解析）

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Abbreviation

2-OG: 2-Oxoglutarate

DW: dry weight

GDH: Glutamate dehydrogenase

gdhA: fungal GDH gene

Gln: gluramate

Glu: glutamate

GOGAT: Glutamate synthase

GS: Glutamine synthetase

N: nitrogen

PCR: polymerase chain reaction

Pn: net photosynthesis rate

PPFD: photosynthetic photon flux density

qRT-PCR: quantitative reverse-transcription PCR

RuBisCO: ribulose 1,5-bisphosphate carboxylase/oxtgenase

CT: control rice plants

General Introduction

Continuous growths of the global population, and the subsequent increase in food demand, have led to a demand for increases in agricultural production. To meet this increased demand, current agricultural practices rely heavily on nitrogenous fertilizers in the form of ammonium (NH_4^+) or nitrate (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 such health problems 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 more effectively 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 NH_4^+. Assimilation of 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 and Miflin, 1974). The ATP-dependent condensation of 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), which has been considered to be important in nitrogen assimilation in higher plants, has been a matter of controversy in NH_4^+ assimilation. GDH catalyzes the reversible amination of 2-OG with 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 NH_4^+ (Harrison et al., 2000). However, in microorganisms such as bacteria or Ascomycota, both NADP(H)-GDH and GS play important roles in NH_4^+ assimilation (Kinghorn and Pateman, 1973). Some reports show that the affinity of GDH for NH_4^+ is considerably higher in microorganisms than in higher plants (Miflin and Lea, 1980; Srivastava and Singh, 1987; Wang and Tian, 2001; Noor and Punekar, 2005). 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 and 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 NH_4^+ assimilation in microorganisms. The presence of NADP(H)-GDH in microorganisms 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 and Kida, 2003), some of which had enhanced biomass and yield. Kisaka and Kida (2003) reported that in tobacco 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. By 2009, 35% of all paddy fields in Japan had been abandoned (MAFF, 2011). A shift from rice to alternative crops such as soybean, wheat and buckwheat has been promoted by the Japanese Government. Many paddy fields are not suitable for upland crops owing to difficulty in draining excess water from the soil. Recently, rice has been used for feeding livestock as well as for human food. Forage rice is an alternative that can adequately maintain productivity of paddy fields (Asada et al., 2013). 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 yield 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. On the other hand, 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. According to previous reports, transgenic plants expressing fungal or bacterial GDHs can show improved drought or herbicide tolerance (Ameziane et al., 2000; Nolte et al., 2004; Lightfoot et al., 2007; Du et al., 2014). Therefore, the introduction of NADP(H)-GDH from microorganisms could improve plant tolerance under stress conditions. Of feed rice, cv. Momiroman seems to be the 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).

Based on these backgrounds, this thesis consists of four chapters to examine 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 chapter 1, transgenic rice plants expressing *gdhA* (cv.Leafstar, Yamahoshi and Momiroman) were generated for the selection of plant materials for further analyses. In chapter 2, the effect of *gdhA* expression in rice was investigated. In details, GDH activities, plant growth, dry weight, nitrogen content and nitrogen uptake efficiency were measured and analyzed at the seedling stage. Furthermore, in chapter 3, the Pn, grain weight, nitrogen content and uptake efficiency were measured and analyzed at the harvest stage. In chapter 4, to examine the tolerance of transgenic rice plants to salt, we examined the effect of exogenously introduced NADP(H)-GDH on salt tolerance capacity of the control and transgenic plants.

Chapter one: Generation of transgenic rice plants expressing *gdhA* and variety selection

1-1. Introduction

GDH catalyzes the reversible amination of 2-oxoglutarate (2-OG) with NH_4^+ to form glutamate in the presence of NAD(P)H as a cofactor, directly connected to the TCA cycle and thereby plays a key role by providing a link between carbon and nitrogen metabolism (Fig. 1-1) (Lea and Miflin, 1974). Although GDHs are often thought to play a key role by providing a link between carbon and nitrogen metabolism, the physiological role(s) of GDH has still remained obscure. On the other hand, some attempts have been made to reinforce GDH activities in plants by overexpressing bacterial/fungal/plant GDH genes. For example, Abiko et al. (2010) analyzed transgenic rice plants (cv. Yamahoushi) expressing a fungal GDH gene, and suggested that the introduction of fungal GDH gene into the food rice cultivar could lead to better growth and higher grain yield by enhancing the assimilation of ammonium. 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 this chapter, a fungal GDH gene was introduced into two rice cultivars Momiroman and Leafstar, and three transgenic lines of each variety were selected and used for further analyses based on the results of genomic PCR and quantitative RT-PCR. Then，the GDH-introduced lines of Momiroman, Leafstar and previously-established GDH-introduced Yamahoushi were subjected to pot experiments, using the original

cultivars as the controls.

1-2. Materials and Methods

1-2-1. Generation of transgenic rice plants

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 (EF1β; Gene Locus Os04g0118400) and a rice prolamin 10 terminator (P10; Os03g0766000). PCR-amplified cDNA for *gdhA*, DNA for the EF1βpromoter and DNA for the P10terminator were subcloned appropriately into a plasmid vector, pUC19 (Takara Bio, Shiga, Japan). The resultant EF1βpromoter–*gdhA*–P10terminator 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 (Fig. 1-2 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 and Leafstar 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 of each variety were selected for further analyses, based on the results of genomic PCR and quantitative RT-PCR analysis (Figs. 1-2 b, c and

1-3) and the abundance of seeds produced. Previously-established Yamahoushi were detected by genomic PCR and quantitative RT-PCR (Fig.1-4).

1-2-2. Plant materials and growth

Seeds were selected by sedimentation in salt solution of specific gravity 1.06, and then washed by water to remove the salt solution. The selected seeds were treated for 10 min in distilled water adjusted to 60 ℃ with gentle shaking, and then soaked for 20 min in distilled water. The seeds were then soaked for 30 s in 70% (v/v) ethanol, and then washed with distilled water. The seeds were soaked for 20 min in 1% (v/v) sodium hypochlorite solution and washed with distilled water. The seeds were germinated in distilled water at 30 ℃ for 48 h. The eleven transgenic seeds of Momiroman and Leafstar, three of Yamahoshi as well as corresponding control, were planted in pots in a greenhouse. The temperature was set to 25 ℃ during the day and 20 ℃ at night with 60% relative humidity under natural light conditions. The rice were cultivated in 1/2000-are pots with soil. Under the high nitrogen condition, 5 g of nitrogen, 5 g of phosphoric acid and 5 g of potassium per pot were added as basal fertilizer. Under the low nitrogen condition, 1 g of nitrogen, 5 g of phosphoric acid and 5 g of potassium per pot were added as basal fertilizer. Four-week-old plants were sampled and the samples for RT-PCR were divided into leaf blades, stems and roots and immediately frozen in liquid nitrogen and kept at –80 ℃ until use. The samples for biomass were divided into leaf blades, stems and roots.

1-2-3. Genomic RNA and RT-PCR analyses of transgenic rice plants

Genomic DNA was extracted from the leaves of control and transgenic lines of three varieties by a modified cetyltriethyl ammnonium bromide method (Murray and Thompson,

1980). For Momiroman and Leafstar, amplification by PCR was carried out using two pairs of primers, *gdhA*-specific primers: 5′-TCCATCCTCAAGTTCCTTGG-3′ and 5′-CGGAAACTTCGTTCTGGGTA-3′; CSP- and mALS-specific primers: 5′-ATCGGCATCGAGACCTATCC-3′ and 5′-TGTCGCTGGTGGTTCTTACG-3′, with 35 cycles of incubation at 94℃ for 30 s, 58℃ for 90 s and 72℃ for 90 s, with a final extension at 72℃ for 5 min. For Yamahoshi, amplification by PCR was carried out using two pairs of primers, *gdhA*-specific primers, and hygromycin resistance gene (*HPT*)-specific primers: 5′-ACATTGTTGGAGCCGAAATC-3′ and 5′-GTGTCACGTTGCAAGACCTG-3′.

Total RNA was extracted from the fully expanded, upper-most leaves of 4-week-old seedlings. 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 mixtures was used as the template for real-time quantitative PCR analysis. Each PCR (10μL of total volume) was performed with 1μL of cDNA mixtures, 5 μL 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℃ for 2 min, 95℃ for 2 min and 40 cycles of 95℃ for 15 s and 60℃ for 31 s. The amount of product was quantified using a standard curve after

normalization with transcripts from an internal control gene *RUBIQ1* (Os06g0681400).

1-2-4. Measurement of plant height, root length, leaf area and dry weight

Four-week-old seedlings were harvested and plant height, root length and leaf area were determined. Leaf blades, stems and roots were separated and dried at 80℃ for 3 d. Dried samples were weighed and ground with Multi-Beads Shocker (Yasui-Kikai, Osaka, Japan).

Measurement of chlorophyll concentration, relative RuBisCO concentration and soluble protein concentration

Frozen upper-most, fully expanded leaf blades of 4-week-old seedlings were homogenized using a chilled mortar and pestle on ice with 1 ml of extraction buffer containing 50 mM HEPES-KOH (pH 7.5), 2 mM EDTA (pH 7.0), 10 mM MgCl, 5 mM DTT, 10% (v ／ v) glycerol and 2% (w ／ v) polyvinylpolypyrrolidone. Some of the homogenates were used for measuring total chlorophyll concentration using dimethylsulphoxide following the method described by Hiscox and Israelstam (1979). The rest of homogenates were centrifuged at 15000 g for 10 min at 4℃, and the supernatants were used for the assessment of RuBisCO concentration according to the methods described by Kanbe et al. (2009); Soluble protein concentration 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.

1-3. Results

1-3-1. Expression level of transgenic rice plants expressing *gdhA*

Transgenic lines of Momiroman and Leafstar expressing *gdhA* under the control of the

EF1βpromoter 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 (Figs. 1-2 b, c and 1-3). Similarly, Transgenic lines of Yamahoshi expressing *gdhA* under the control of the cauliflower mosaic virus (CaMV) 35S promoter were confirmed by PCR analysis using *gdhA* (amplified fragment 711 bp) and *HPT* (the selection marker, amplified fragment 320 bp) specific primers and further identified by semi-quantitative RT-PCR (Fig.1-4). These results indicated that *gdhA* was successfully introduced and expressed in transgenic rice plants.

1-3-2. Effect of introduction of a fungal *gdhA* into Momiroman

1-3-2-1. Effect of *gdhA* expression on growth and dry weight at the seedling stage

Plant growth analysis at the seedling stage revealed that the plant height, root length and leaf area of TG 7 and TG 10 were significantly higher than those of control line in both high and low N plants, while the root length and leaf area of TG 5 were significantly higher than those of control line in low N plants (Figs. 1-5, 1-6 and 1-7). The leaf, stem and root dry weights of all transgenic lines were higher than those of control line, regardless of the nitrogen conditions (Fig. 1-8).

1-3-2-2. Effect of *gdhA* expression on chlorophyll concentration, relative RuBisCO concentration and soluble protein concentration at the seedling stage

The chlorophyll concentration of all transgenic lines was higher than control line, although only TG 7 and TG 10 were significantly higher than control line under both high and low N conditions (Fig. 1-9). The relative RuBisCO concentration and soluble protein concentration of TG 7 and TG 10 lines were significantly higher than those of control line,

regardless of the nitrogen conditions (Figs. 1-10 and 1-11). The relative RuBisCO concentration and soluble protein concentration of TG 5 line were slightly higher than control line under high N conditions, but were opposite under low N conditions (Figs. 1-10 and 1-11).

1-3-3. Effect of introduction of a fungal *gdhA* into Yamahoshi

1-3-3-1. Effect of *gdhA* expression on growth and dry weight at the seedling stage

The plant height, root length and leaf area of transgenic lines did not differ from control line except the leaf area of TG 30 was higher than control line under low N condition (Figs. 1-12, 1-13 and 1-14). The leaf, stem and root dry weights of TG 30 were higher than those of control line in both high and low N plants, the TG 3 was also higher than control line under high N condition. However, the TG 13 was lower than control line under low N condition (Fig. 1-15).

1-3-3-2. Effect of *gdhA* expression on chlorophyll concentration, relative RuBisCO concentration and soluble protein concentration at the seedling stage

The chlorophyll concentration, relative RuBisCO concentration and soluble protein concentration of TG 13 line were significantly higher than control line in both high and low N plants except the relative RuBisCO concentration was slightly higher than control line under high N condition (Figs. 1-16, 1-17 and 1-18). The relative RuBisCO concentration of TG 3 lines was also higher than control line under low N condition (Fig. 1-17).

1-3-4. Effect of introduction of a fungal *gdhA* into Leafstar

1-3-4-1. Effect of *gdhA* expression on growth and dry weight at the seedling stage

The plant height, root length leaf area and dry weight of all transgenic lines were significantly lower than control line in both high and low N plants except the plant height of

TG 5 and TG 6 did not differ from control line (Figs. 1-19, 1-20, 1-21 and 1-22).

1-3-4-2. Effect of *gdhA* expression on chlorophyll concentration, relative RuBisCO concentration and soluble protein concentration at the seedling stage

The chlorophyll concentration of all transgenic lines was lower than control line under both high and low N conditions (Fig. 1-23). The relative RuBisCO concentration of TG 5 under low N condition and TG 6 under high N condition was significantly lower than control line (Fig. 1-24). The soluble protein concentration of all transgenic lines was lower than control line under low N condition, and all transgenic lines did not differ from control line under high N condition (Fig. 1-25).

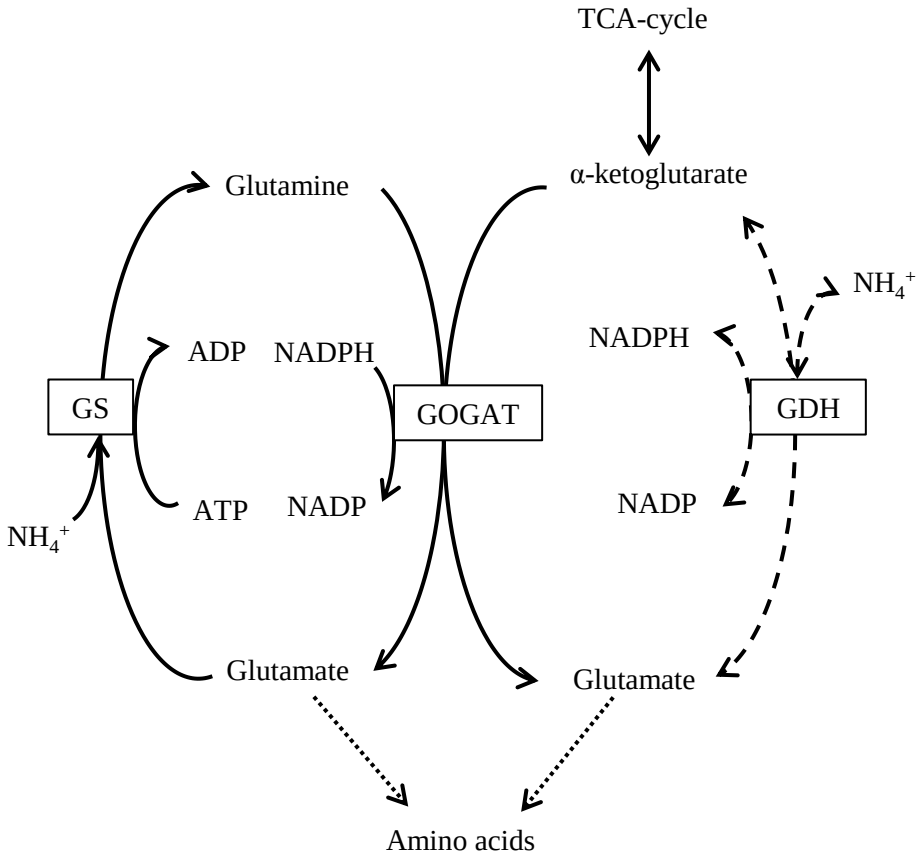


Fig. 1-1. The pathway of ammonia assimilation in plants. Broken line shows the possible nitrogen assimilation pathway added by the introduction of fungal NADP(H)-GDH.

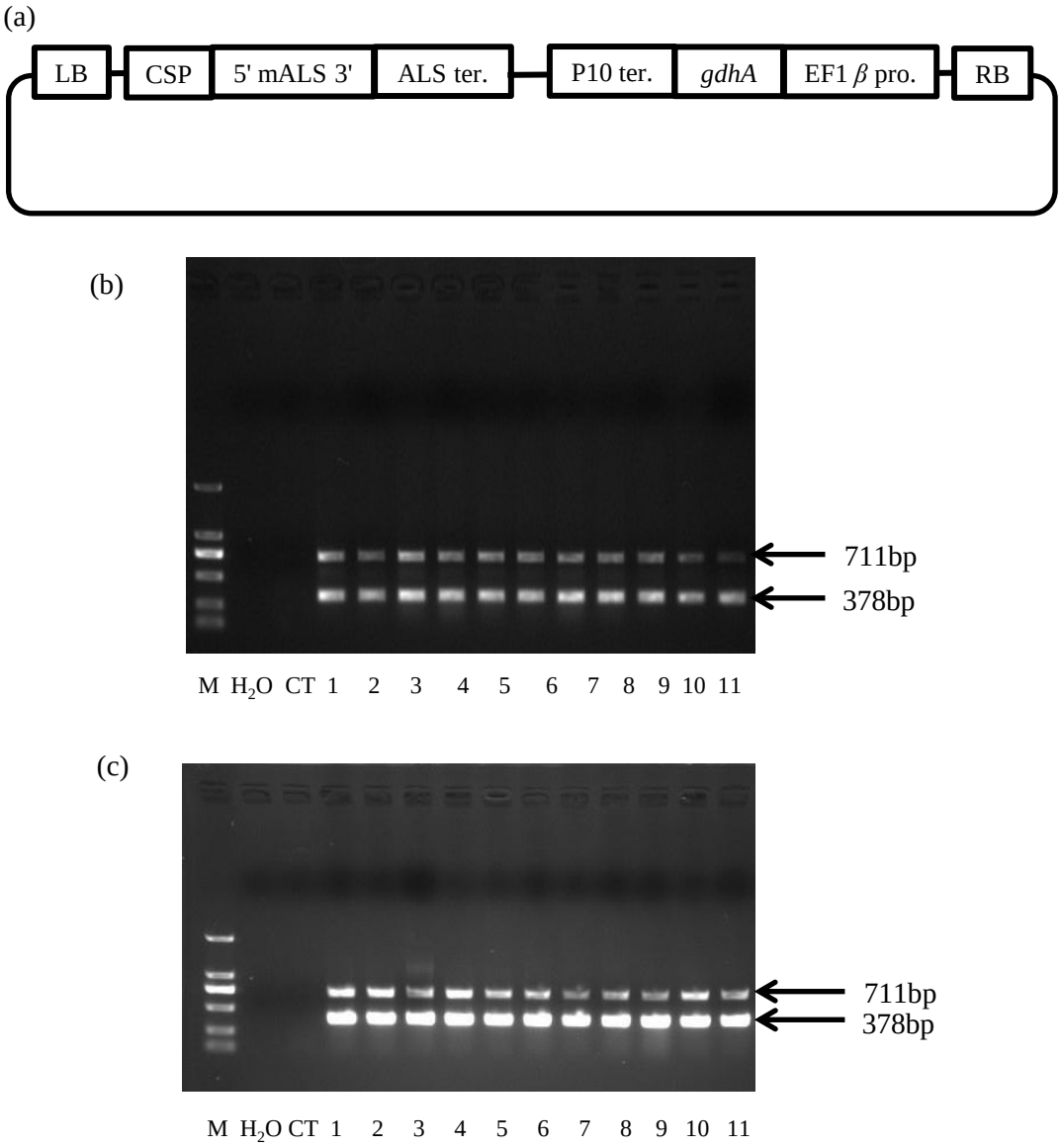


Fig. 1-2. Generation of transgenic rice plants expressing the *gdhA* gene. (a) Plasmid construct for the transformation. LB, left border; RB, right border; CSP, callus specific promoter; mALS, mutated acetolactate synthase gene; ALS ter., ALS terminator; EF1β pro, elongation factor 1beta promoter; P10 ter, prolamin 10 terminator. (b) and (c) Genomic PCR of control (CT) and transgenic rice plants (b, Momiroman; c, Leafstar), M, Marker; CT, control line; 1-11 lines, transgenic lines. The *gdhA* gene, 711bp; mALS gene, 378bp.

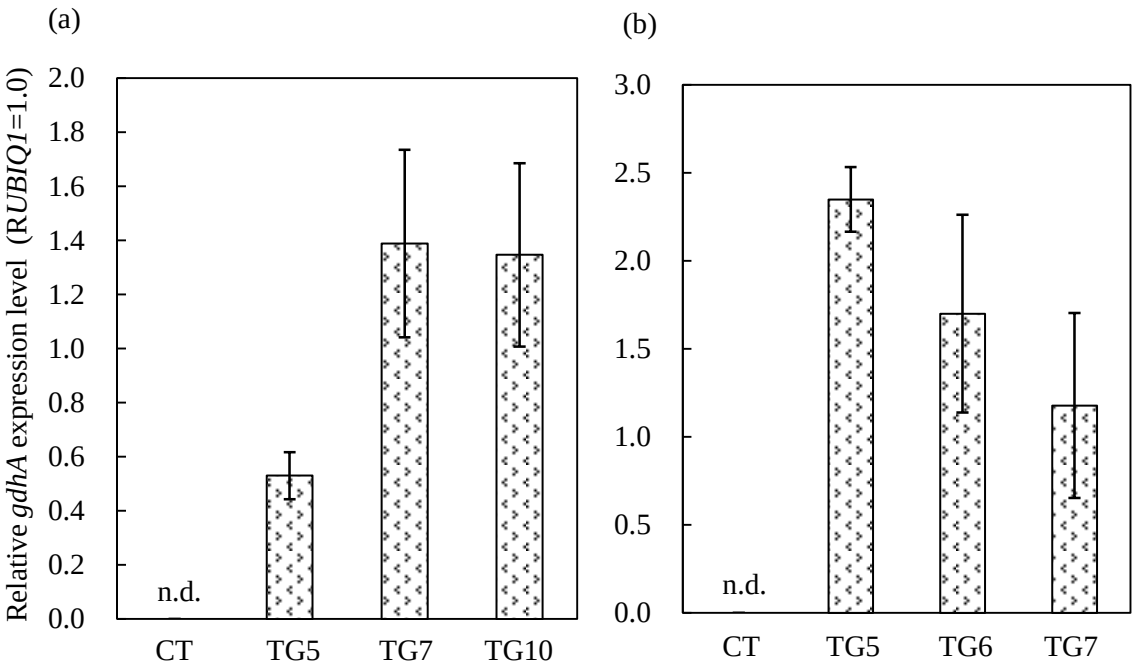


Fig. 1-3. Quantitative RT-PCR of *gdhA* transcripts in the leaves. RT-PCR was performed with RNA from the leaves of the T_3 generations of control (CT) and transgenic (TG) lines (a, Momiroman; b, Leafstar). Values are expressed as the means ± SD of eight replicates, after normalization with internal control (*RUBIQ1*). The letters 'n.d.' indicates 'not detected'.

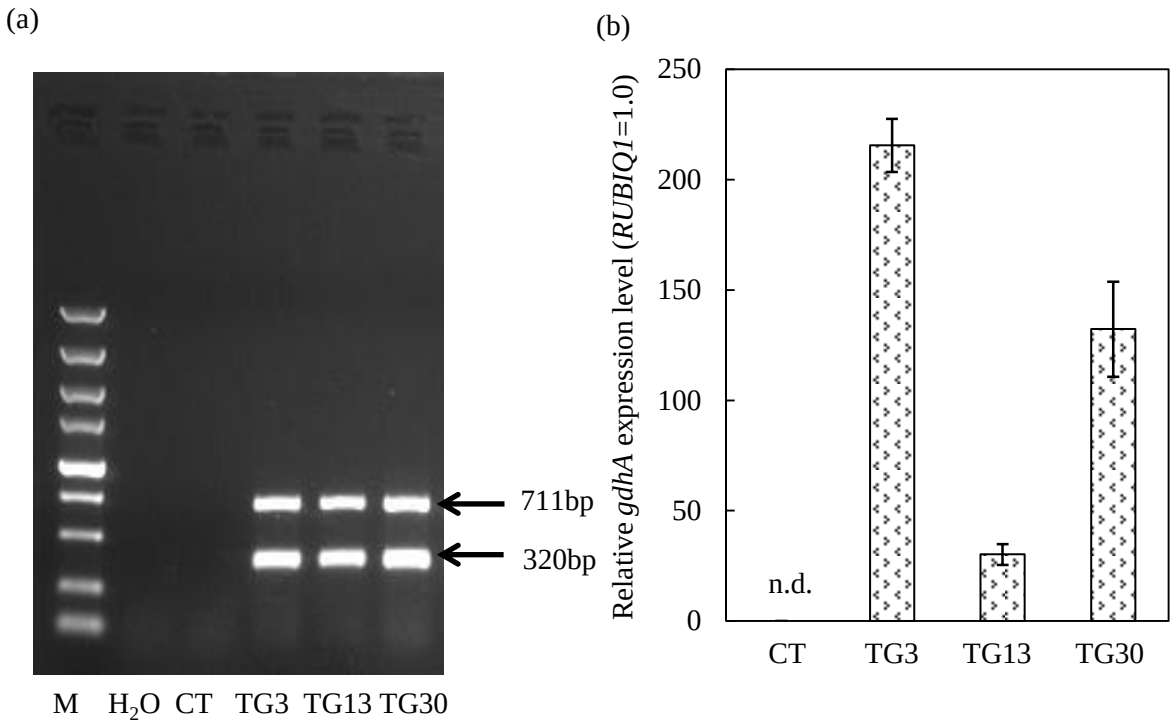


Fig. 1-4. Generation of transgenic rice plants of Yamahoshi expressing the *gdhA* gene. (a) PCR analysis of transgenic rice plants, M: Marker; CT: control line; TG: transgenic lines. The *gdhA* gene, 711bp; HPT (selection marker) gene, 320bp. (b) Quantitative RT-PCR of *gdhA* transcripts in the leaves of Yamahoshi. RT-PCR was performed with RNA from the leaves of CT and three *gdhA* rice lines. Values are expressed as the means ± SD of eight replicates, after normalization with internal control (*RUBIQ1*). The letters 'n.d.' indicates 'not detected'.

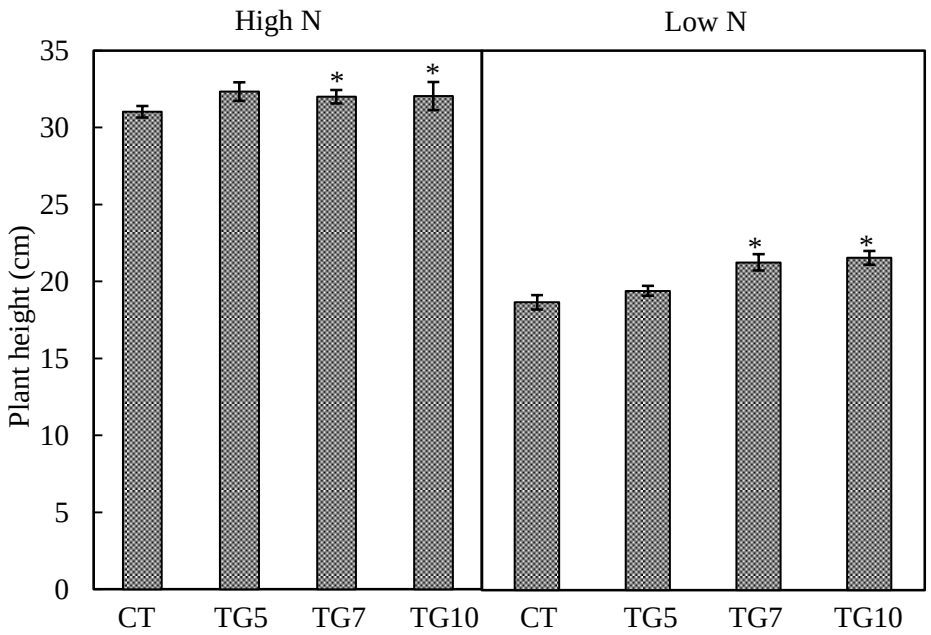


Fig. 1-5. Plant height of control (CT) and transgenic (TG5, TG7, TG10) lines of Momiroman under high and low N treatment conditions at seedling stage. Data represent the mean values ± SD (n = 9). Statistical analysis of the data was performed by one-way ANOVA. Asterisks indicate that the mean values of TG7 and TG10 lines are significantly different from that of CT at $p < 0.05$ (*).

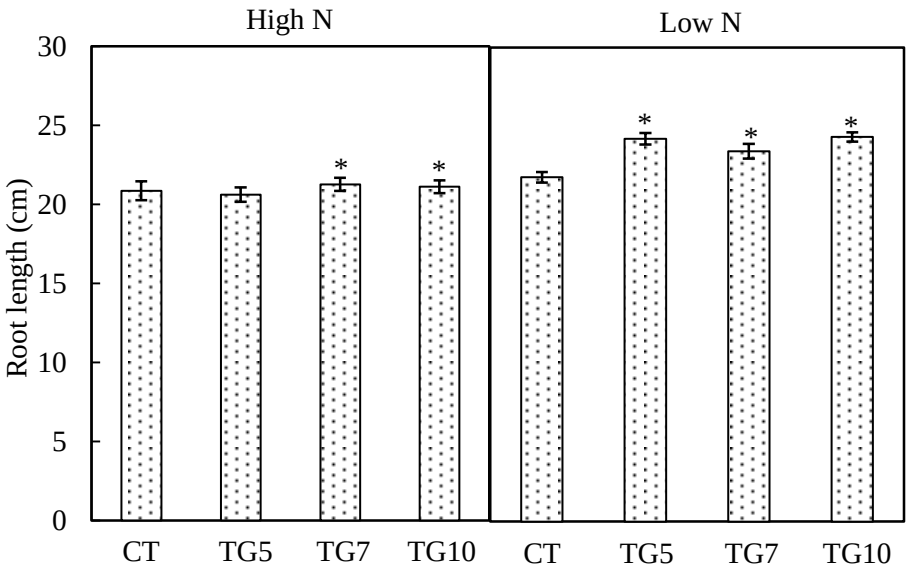


Fig. 1-6. Root length of control (CT) and transgenic (TG5, TG7, TG10) lines of Momiroman under high and low N treatment conditions at seedling stage. Data represent the mean values ± SD (n = 9). Statistical analysis of the data was performed by one-way ANOVA. Asterisks indicate that the mean values of TG5, TG7 and TG10 lines are significantly different from that of CT at $p < 0.05$ (*).

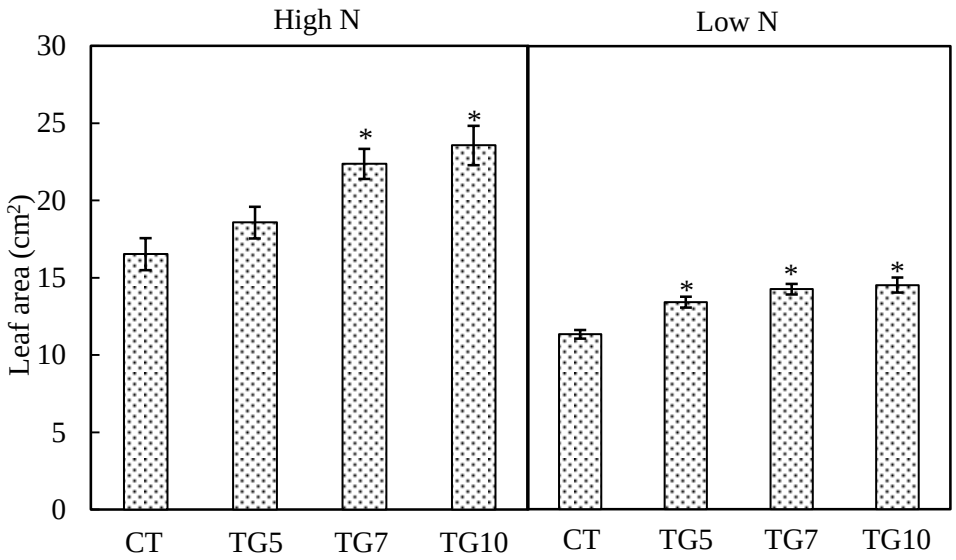


Fig. 1-7. Leaf area of control (CT) and transgenic (TG5, TG7, TG10) lines of Momiroman under high and low N treatment conditions at seedling stage. Data represent the mean values ± SD (n = 9). Statistical analysis of the data was performed by one-way ANOVA. Asterisks indicate that the mean values of TG5, TG7 and TG10 lines are significantly different from that of CT at $p < 0.05$ (*).

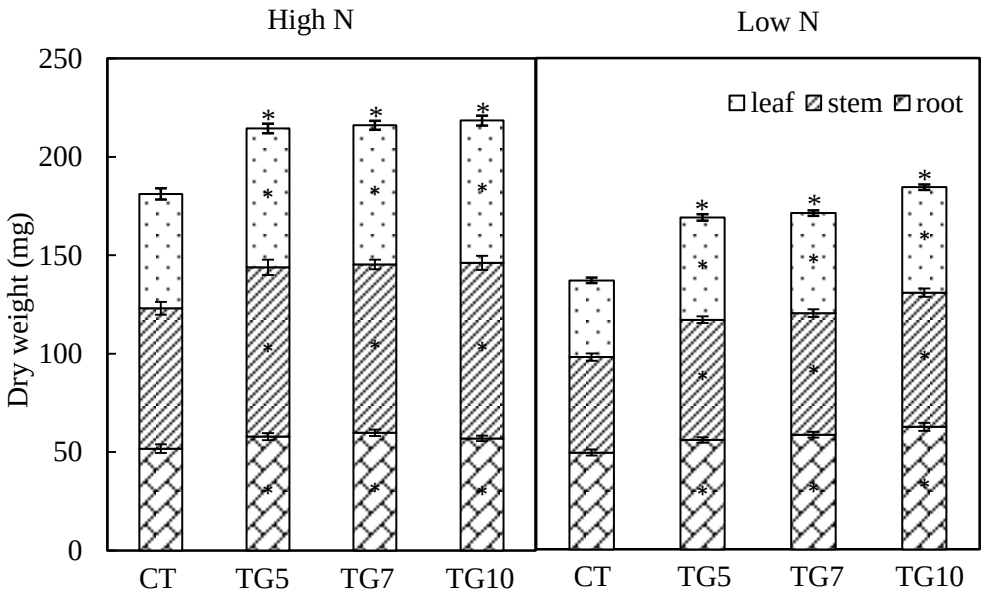


Fig. 1-8. Dry weight of control (CT) and transgenic (TG5, TG7, TG10) lines of Momiroman under high and low N treatment conditions at seedling stage. Data represent the mean values ± SD (n = 9). Statistical analysis of the data was performed by one-way ANOVA. Asterisks indicate that the mean values of TG5, TG7 and TG10 lines are significantly different from that of CT at $p < 0.05$ (*).

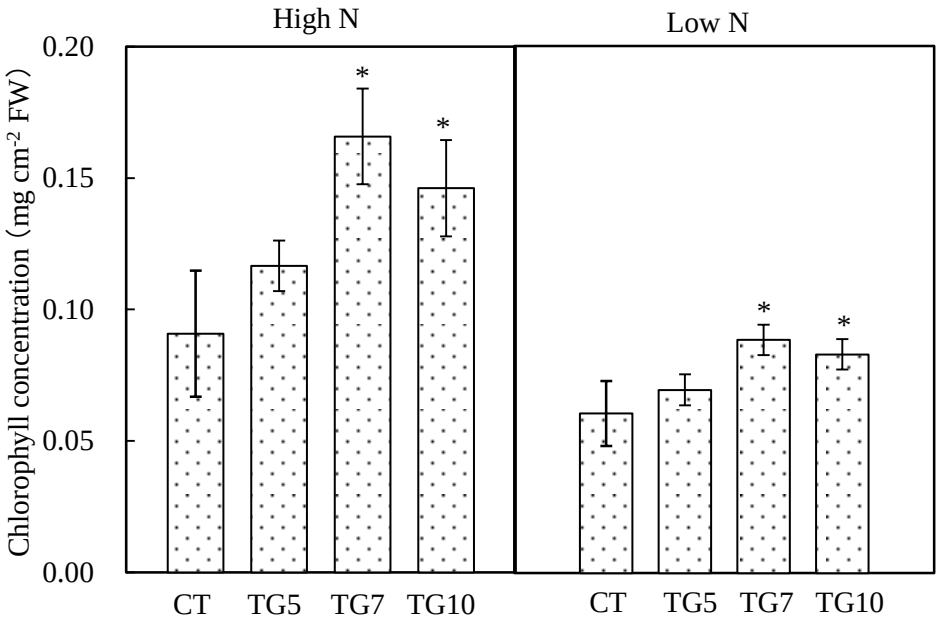


Fig. 1-9. Chlorophyll concentration of control (CT) and transgenic (TG5, TG7, TG10) lines of Momiroman under high and low N treatment conditions at seedling stage. Data represent the mean values ± SD (n = 4). Statistical analysis of the data was performed by one-way ANOVA. Asterisks indicate that the mean values of TG7 and TG10 lines are significantly different from that of CT at $p < 0.05$ (*).

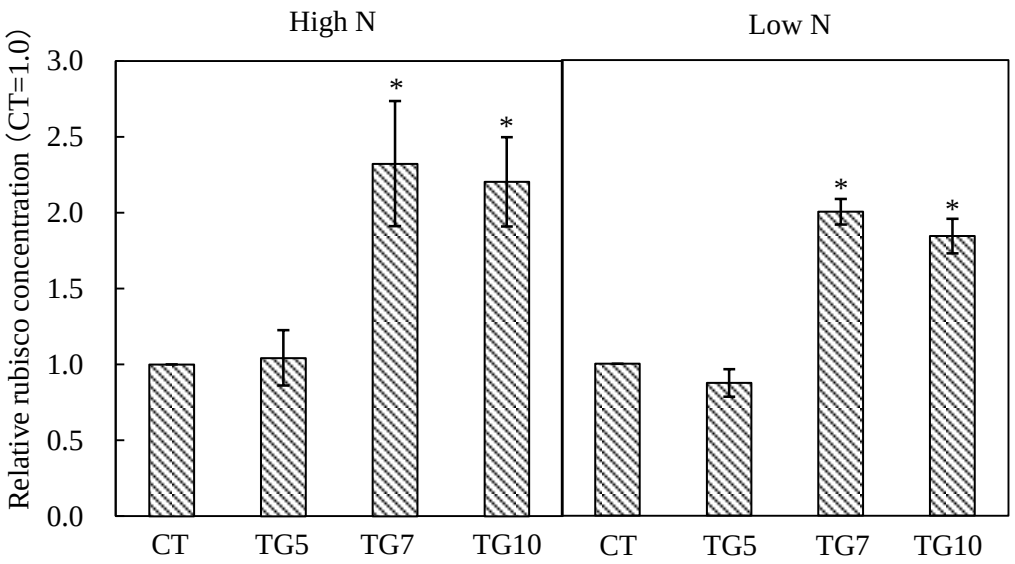


Fig. 1-10. Relative rubisco concentration of control (CT) and transgenic (TG5, TG7, TG10) lines of Momiroman under high and low N treatment conditions at seedling stage. Data represent the mean values ± SD (n = 4). Statistical analysis of the data was performed by one-way ANOVA. Asterisks indicate that the mean values of TG7 and TG10 lines are significantly different from that of CT at $p < 0.05$ (*).

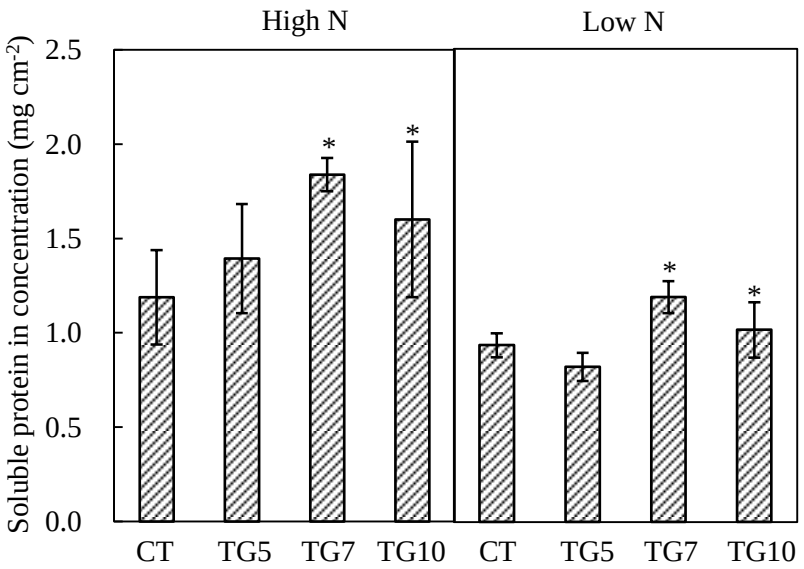


Fig. 1-11. Soluble protein concentration of control (CT) and transgenic (TG5, TG7, TG10) lines of Momiroman under high and low N treatment conditions at seedling stage. Data represent the mean values ± SD (n = 4). Statistical analysis of the data was performed by one-way ANOVA. Asterisks indicate that the mean values of TG7 and TG10 lines are significantly different from that of CT at $p < 0.05$ (*).

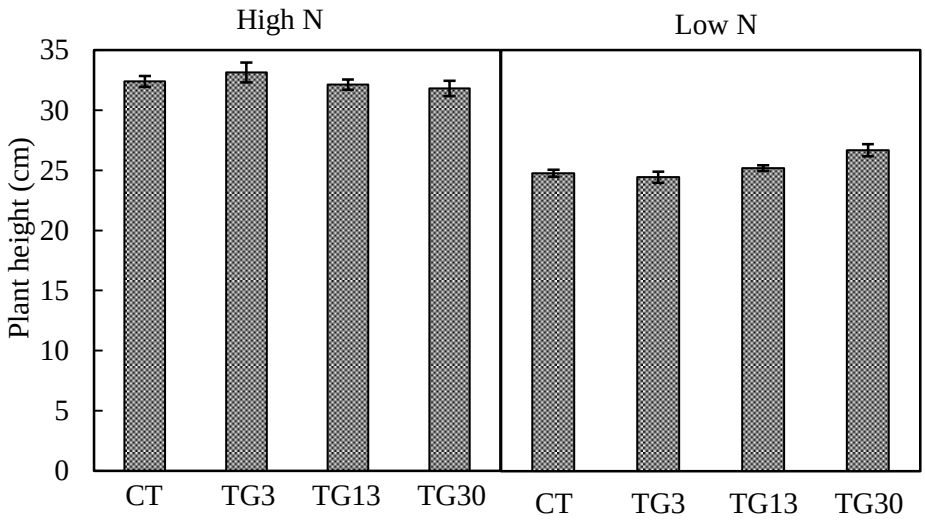


Fig. 1-12. Plant height of control (CT) and transgenic (TG3, TG13, TG30) lines of Yamahoshi under high and low N treatment conditions at seedling stage. Data represent the mean values ± SD (n = 9). Statistical analysis of the data was performed by one-way ANOVA.

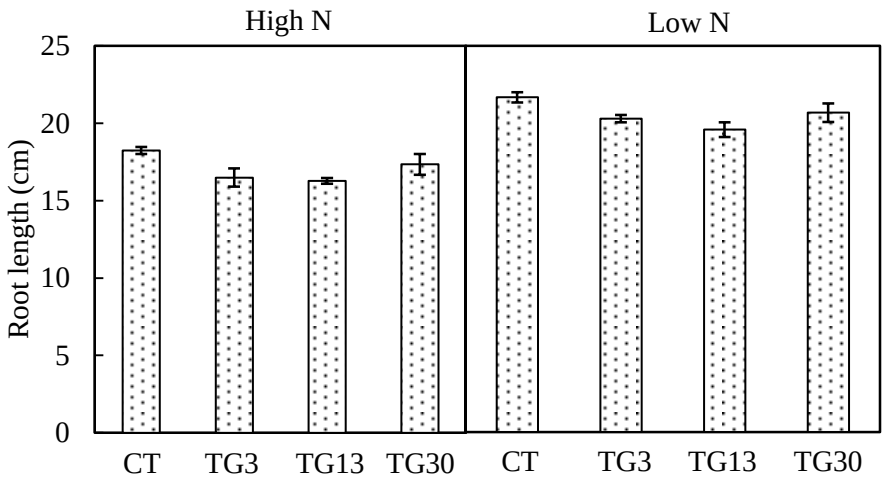


Fig. 1-13. Root length of control (CT) and transgenic (TG3, TG13, TG30) lines of Yamahoshi under high and low N treatment conditions at seedling stage. Data represent the mean values ± SD (n = 9). Statistical analysis of the data was performed by one-way ANOVA.

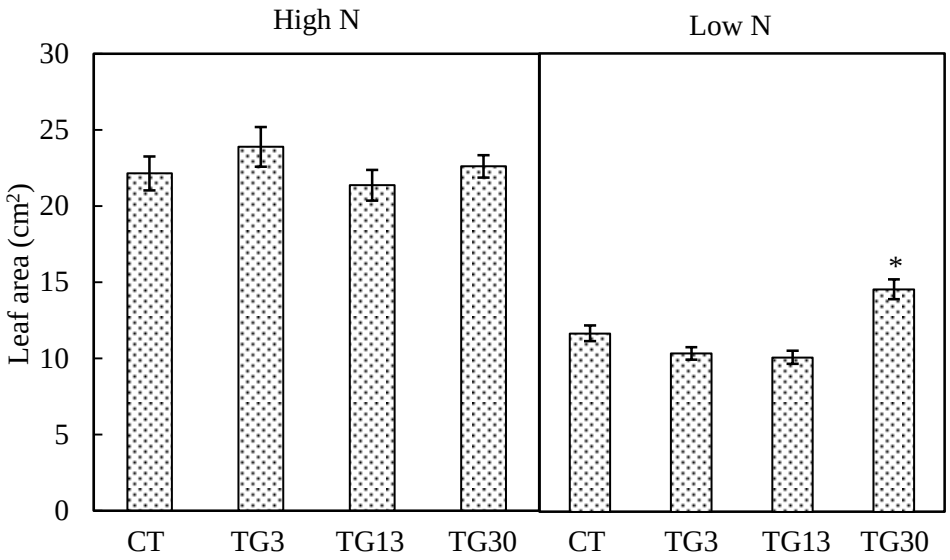


Fig. 1-14. Leaf area of control (CT) and transgenic (TG3, TG13, TG30) lines of Yamahoshi under high and low N treatment conditions at seedling stage. Data represent the mean values ± SD (n = 9). Statistical analysis of the data was performed by one-way ANOVA. Asterisks indicate that the mean values of TG30 lines are significantly different from that of CT at $p < 0.05$ (*).

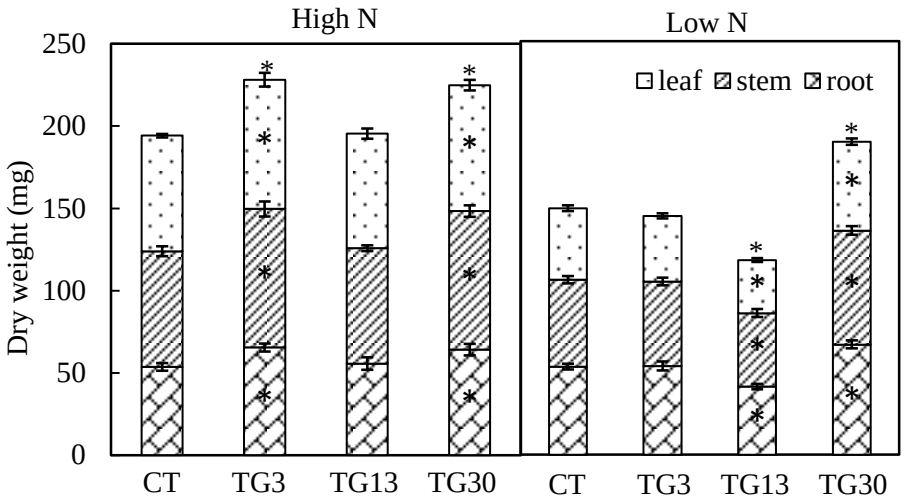


Fig. 1-15. Dry weight of control (CT) and transgenic (TG3, TG13, TG30) lines of Yamahoshi under high and low N treatment conditions at seedling stage. Data represent the mean values ± SD (n = 9). Statistical analysis of the data was performed by one-way ANOVA. Asterisks indicate that the mean values of TG3, TG13 and TG30 lines are significantly different from that of CT at $p < 0.05$ (*).

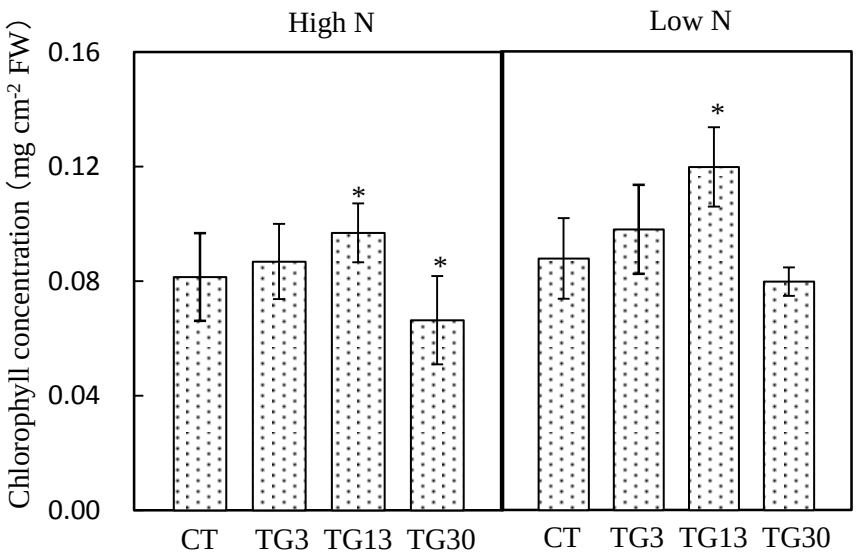


Fig. 1-16. Chlorophyll concentration of control (CT) and transgenic (TG3, TG13, TG30) lines of Yamahoshi under high and low N treatment conditions at seedling stage. Data represent the mean values ± SD (n = 4). Statistical analysis of the data was performed by one-way ANOVA. Asterisks indicate that the mean values of TG13 and TG30 lines are significantly different from that of CT at $p < 0.05$ (*).

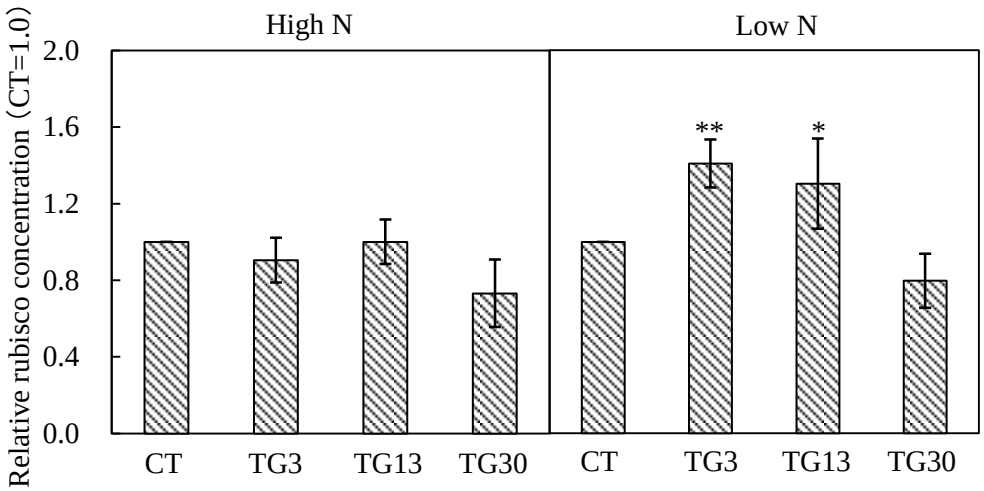


Fig. 1-17. Relative rubisco concentration of control (CT) and transgenic (TG3, TG13, TG30) lines of Yamahoshi under high and low N treatment conditions at seedling stage. Data represent the mean values ± SD (n = 4). Statistical analysis of the data was performed by one-way ANOVA. Asterisks indicate that the mean values of TG3 and TG13 lines are significantly different from that of CT at $p < 0.05$ (*) and $p < 0.01$ (**).

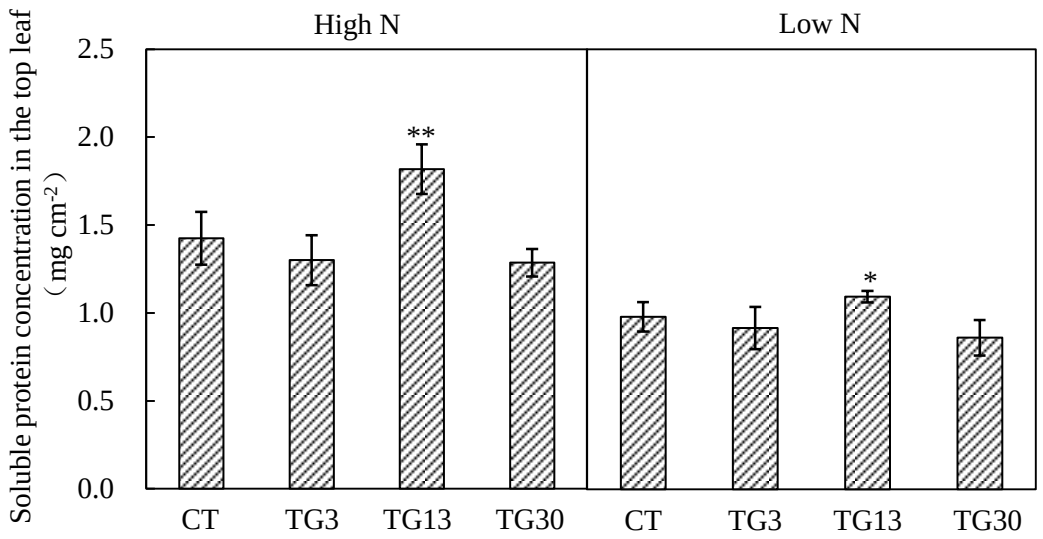


Fig. 1-18. Soluble protein concentration of control (CT) and transgenic (TG3, TG13, TG30) lines of Yamahoshi under high and low N treatment conditions at seedling stage. Data represent the mean values ± SD (n = 4). Statistical analysis of the data was performed by one-way ANOVA. Asterisks indicate that the mean values of TG13 lines are significantly different from that of CT at $p < 0.05$ (*) and $p < 0.01$ (**).

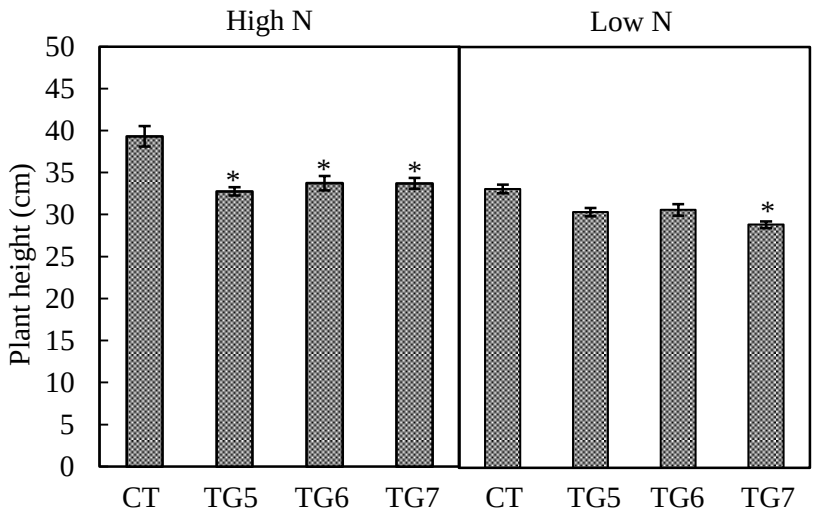


Fig. 1-19. Plant height of control (CT) and transgenic (TG5, TG6, TG7) lines of Leafstar under high and low N treatment conditions at seedling stage. Data represent the mean values ± SD (n = 9). Statistical analysis of the data was performed by one-way ANOVA. Asterisks indicate that the mean values of TG5, TG6 and TG7 lines are significantly different from that of CT at $p < 0.05$ (*).

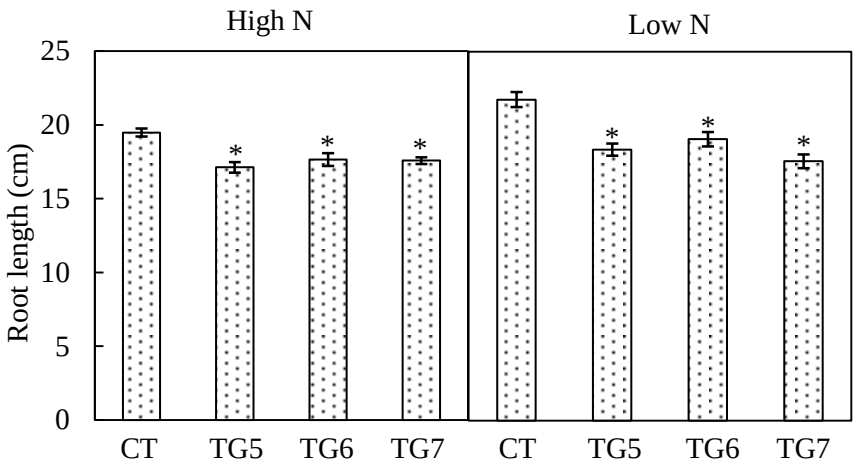


Fig. 1-20. Root length of control (CT) and transgenic (TG5, TG6, TG7) lines of Leafstar under high and low N treatment conditions at seedling stage. Data represent the mean values ± SD (n = 9). Statistical analysis of the data was performed by one-way ANOVA. Asterisks indicate that the mean values of TG5, TG6 and TG7 lines are significantly different from that of CT at $p < 0.05$ (*).

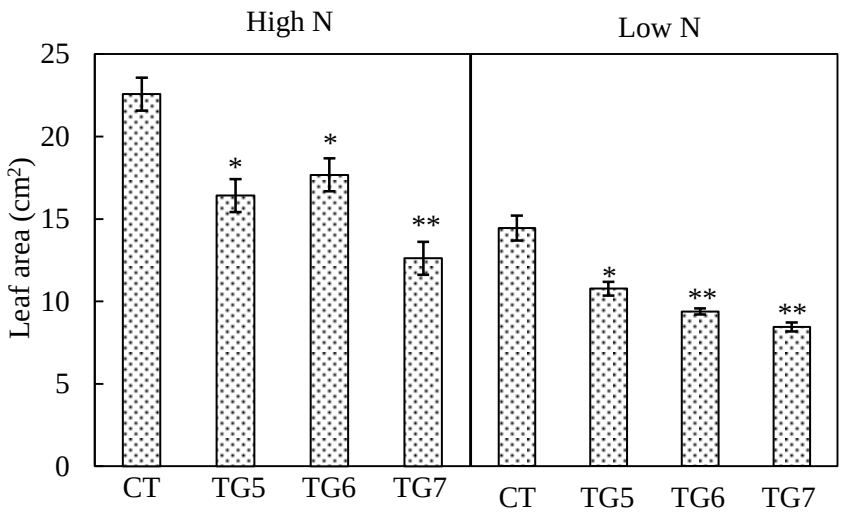


Fig. 1-21. Leaf area of control (CT) and transgenic (TG5, TG6, TG7) lines of Leafstar under high and low N treatment conditions at seedling stage. Data represent the mean values ± SD (n = 9). Statistical analysis of the data was performed by one-way ANOVA. Asterisks indicate that the mean values of TG5, TG6 and TG7 lines are significantly different from that of CT at $p < 0.05$ (*) and $p < 0.01$ (**).

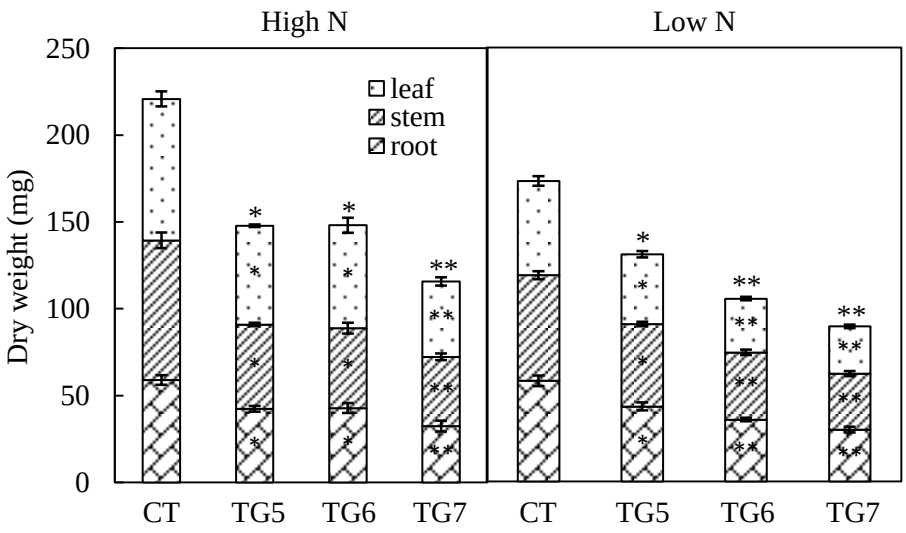


Fig. 1-22. Dry weight of control (CT) and transgenic (TG5, TG6, TG7) lines of Leafstar under high and low N treatment conditions at seedling stage. Data represent the mean values ± SD (n = 9). Statistical analysis of the data was performed by one-way ANOVA. Asterisks indicate that the mean values of TG5, TG6 and TG7 lines are significantly different from that of CT at $p < 0.05$ (*) and $p < 0.01$ (**).

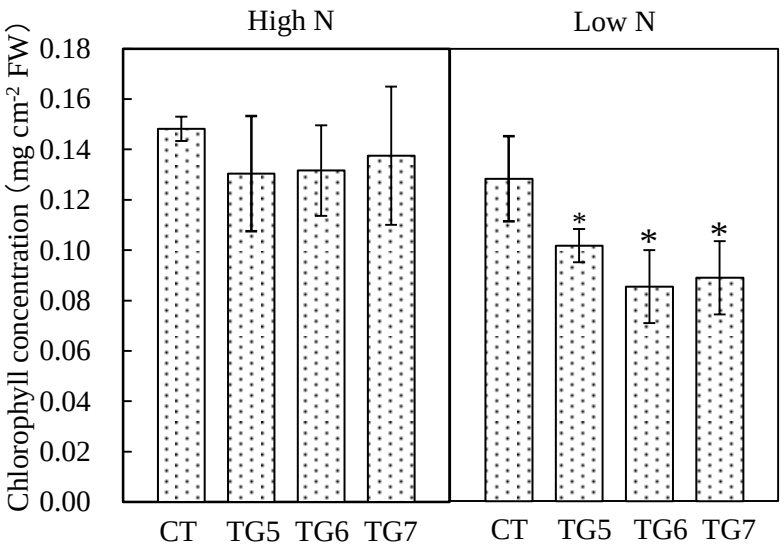


Fig. 1-23. Chlorophyll concentration of control (CT) and transgenic (TG5, TG6, TG7) lines of Leafstar under high and low N treatment conditions at seedling stage. Data represent the mean values ± SD (n = 4). Statistical analysis of the data was performed by one-way ANOVA. Asterisks indicate that the mean values of TG5, TG6 and TG7 lines are significantly different from that of CT at $p < 0.05$ (*).

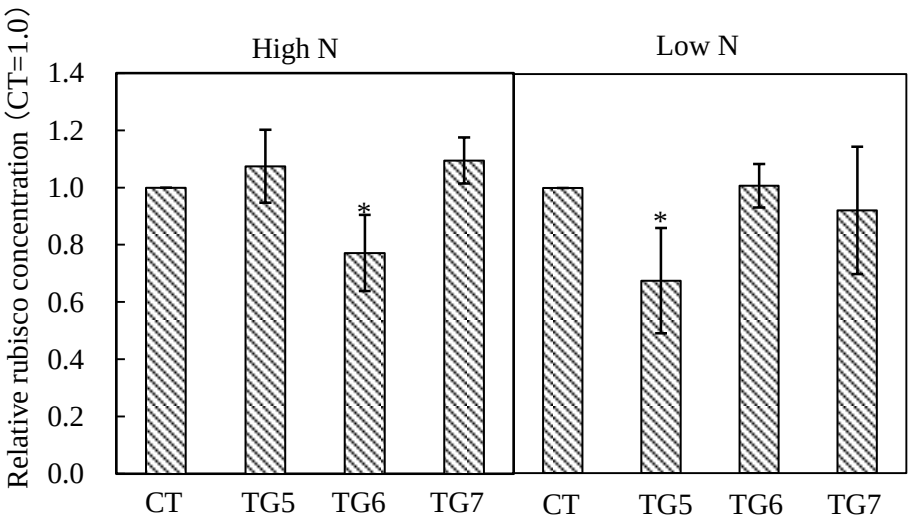


Fig. 1-24. Relative rubisco concentration of control (CT) and transgenic (TG5, TG6, TG7) lines of Leafstar under high and low N treatment conditions at seedling stage. Data represent the mean values ± SD (n = 4). Statistical analysis of the data was performed by one-way ANOVA. Asterisks indicate that the mean values of TG5 and TG6 lines are significantly different from that of CT at $p < 0.05$ (*).

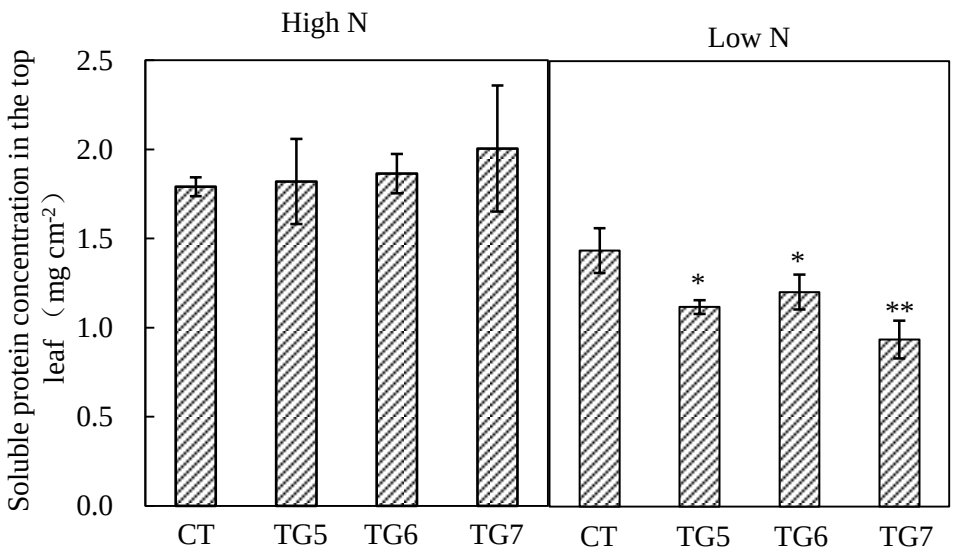


Fig. 1-25. Soluble protein concentration of control (CT) and transgenic (TG5, TG6, TG7) lines of Leafstar under high and low N treatment conditions at seedling stage. Data represent the mean values ± SD (n = 4). Statistical analysis of the data was performed by one-way ANOVA. Asterisks indicate that the mean values of TG5, TG6 and TG7 lines are significantly different from that of CT at $p < 0.05$ (*) and $p < 0.01$ (**).

1-4. Discussion

In this study, we successfully produced transgenic forage rice plants (Momiroman and Leafstar) that expressed a fungal NADP(H)-GDH (*gdhA*) under the control of a constitutive EF1βprom (Fig. 1-2 a). Root system development and physiological activity of roots affect nitrogen uptake in rice plants significantly (Ookawa etal., 2003). In plants with well-developed root systems, increased nitrogen accumulation from roots to shoot have been reported to maintain photosynthesis in rice (Jiang et al., 1988b; Soejima et al., 1995), wheat (Nakamura et al., 2003; Nakagami et al., 2004) and maize (Ma and Dwyer, 1998; Kondo et al., 2000; Fujita et al., 2002). The present study showed that the root length of two transgenic lines (TG 7 and TG 10) of Momiroman were significantly higher than control line (Fig. 1-6); and the root dry weight of all TG lines of Momiroman were also higher than control line under high and low N conditions (Fig. 1-8). In rice, the net photosynthetic rate is closely associated with the level of Rubisco (Makino et al., 1985; Ookawa et al., 2004). Our data indicated that the relative RuBisCO concentration of two transgenic lines (TG 7 and TG 10) of Momiroman were significantly higher than control line (Fig. 1-10). Furthermore, introduction of *gdhA* led to a significant increase in the plant height, leaf area, dry weight of leaf and stem, the chlorophyll concentration and soluble protein concentration in two of transgenic lines (TG 7 and TG 10) of Momiroman under both nitrogen treatment conditions (Figs. 1-5, 1-7, 1-8, 1-9 and 1-11). All these results indicate that transgenic of *gdhA* into forage rice (Momiroman) may increase the net photosynthetic rate and further increases the yield. The leaf, stem and root dry weights of TG 30 of Yamahoshi, which was used for the comparison, were higher than those of control line in both high and low N plants (Fig. 1-15), the chlorophyll concentration, relative RuBisCO concentration and soluble protein concentration of TG 13 line were significantly higher than control in both

high and low N plants (Figs. 1-16, 1-17 and 1-18). However, all the morphology and physiological traits in transgenic plant of Leafstar were significantly lower or not differ from control line (Figs. 1-19, 20, 21, 22, 23, 24 and 25). From this preliminary experiment, the dry matter production of Leafstar was very low, and got many poor qualities of the seeds. But, we reaped a lot of good seeds of Momiroman at the same conditions. Previously our research group already reported expression of a *gdhA* in food rice plants (cv. Yamahoshi) (Abiko et al., 2010). Therefore, we decided to continue this research with Momiroman as forage rice material. In addition, in order to reduce the influence of factors or substances in soil, we conducted hydroponic experiments for further analyses.

Chapter two: Effect of *gdhA* expression on GDH activities, dry weight, nitrogen content and nitrogen uptake efficiency at the seedling stage

2-1. Introduction

Nitrogen is the most important of the mineral nutrients required by plants and its metabolism is tightly coordinated with carbon metabolism in the fundamental processes that permit plant growth (Ishii et al., 2011; Kusano et al., 2011). Nitrogen fertilizers have been extensively used to increase grain yield. However, the current agricultural situation requires that growers must optimize the use of nitrogen fertilizers to avoid pollution. Therefore, exhibiting improved nitrogen uptake efficiency, and adapting agricultural practices to reduce the use of nitrogen fertilizers represents a challenge for both breeders and farmers (Fontaine et al., 2009). In a paddy field, NH_4^+ rather than nitrate NO_3^- tends to be considered the main source of nitrogen for rice, since its metabolism requires less energy than that of NO_3^- (Bloom et al., 1992; Wang et al., 1993). However, in recent years, researchers have paid more and more attention to the partial NO_3^- nutrition of rice crops, when both nitrogen sources are provided simultaneously, growth and yield are often enhanced significantly compared with growth on either NH_4^+ or NO_3^- alone (Kronzucker et al., 1999). The partial replacement of NH_4^+ by NO_3^- could increase the number of the ammonium transporters but did not affect the affinity of the transporters for NH_4^+.

In this chapter, in order to reduce the influence of factors or substances other than that of nitrogen, hydroponic experiments (NH_4NO_3 as nitrogen source) were carried out to study the effects of *gdhA* expression on growth, NADP(H)-GDH activity, dry weight, nitrogen content and uptake efficiency and nitrogen metabolism related gene expression in rice under both high

N and low N conditions were examined. For the determination of nitrogen treatment, our lab had already established the experimental setup for hydroponic culture with nitrogen treatments based on previous studies. That is to perform experiments hydroponically using Yoshida nutrient solution (Yoshida et al., 1976). Our lab had shown that one-quarter strength of Yoshida solution contains enough nutrients to grow rice (Tsunahsima, 2008; Kawakami, 2009). A similar approach was taken by Mae and Ohira (1981), where they grew with stronger nutrient solution. Based on these previous studies, one-quarter strength of Yoshida solution was used as a "high N" condition. In addition, it was also shown that rice seedling grown under one thirty-second strength of Yoshida solution could not survive, but could survive under one sixteenth strength of Yoshida solution (Tsunashima, 2008). Therefore, one sixteenth of Yoshida solution was used as an "low N" condition.

2-2. Materials and Methods

2-2-1. Plant materials and growth

Transgenic and nontransgenic rice plants were grown hydroponically. 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 (Table 2-1) in a greenhouse (day/night cycle of 14/10 h and 25/20 ℃). For high nitrogen (high N) treatment, the Yoshida nutrient solution ingredients were diluted to 1/4-strength, resulting in a final concentration of 360 μM nitrogen (180 μM NH_4NO_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 μM 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 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℃ until use. The samples for biomass analysis were divided into leaf blades, stems and roots.

2-2-2. 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 ammnonium bromide method (Murray and Thompson, 1980). Total RNA was extracted from the fully expanded, upper-most leaves of 6-week-old seedlings. 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 μL of total volume) was performed with 1 μL of cDNA mixtures, 5 μL 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; Glu1 specific primer combination: 5′-AAACAGGCAGCGAGAAAGGT-3′ as the forward and 5′-ACTCGTTCAAACTCGGCACA-3′ as the reverse primer; Gln1;1 specific primer combination: 5′-CAAGTCCGCCATTGAGAAGC-3′ as the forward and 5′-CTTGCCGTTCTGCTCCGTCT-3′ as the reverse primer; Gln1;2 specific primer combination: 5′-GGTTGGAGGATCGGGCATAG-3′ as the forward and 5′-TCACCTTGTGGCGTGTAGCA-3′ as the reverse primer; Gln2 specific primer combination: 5′-ACCAAGAGTATGCGTGAAGA-3′ as the forward and 5′-AACCTGTCAACCTCCTTTCA-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 ℃ for 2 min, 95 ℃ for 2 min and 40 cycles of 95 ℃ for 15 s and 60 ℃ for 31 s. The amount of product was quantified using a standard curve after normalization with transcripts from an internal control gene *RUBIQ1* (Os06g0681400).

2-2-3. 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 ℃. 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 NH_4Cl, 10 mM 2-OG and 0.1 mM β-NADP(H) 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 β-$NADP^+$ at pH 9.3. The reaction was routinely started by the addition of coenzyme (β-NADP(H) or β-$NADP^+$, 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 concentration 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.

2-2-4. 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℃ 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 (Fig. 2-1).

2-2-5. Statistical analysis

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

2-3. Results

2-3-1. GDH activities of transgenic rice plants expressing *gdhA*

As shown in Chapter 1, *gdhA* was successfully introduced and expressed in transgenic rice plants, although the expression level was lower in TG 5 than in TG 7 and TG 10 lines.

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 conditions (Fig. 2-2). 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 TG 7 and TG 10 than in TG5 regardless of nitrogen conditions (Fig. 2-2). We called the former two 'high *gdhA*-expressors' and the latter 'low *gdhA*-expressors'.

2-3-2. 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 μM nitrogen, indicating that there was sufficient nitrogen and other nutrients for the growth of the seedlings. However, under 90 μM nitrogen, control plants exhibited elongated roots and light-green leaves, suggesting that there was insufficient nitrogen for normal growth (Figs. 2-3 and 2-4). Plant growth analysis at the

seedling stage revealed that the plant height, 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*-expressor showed higher dry weight only under high N conditions (Table 2-2, Figs. 2-5 and 2-6). The stem nitrogen concentrations were higher for high *gdhA*-expressors than control line under low N conditions, but were opposite under high N conditions (Fig. 2-7). The nitrogen contents in leaves, stems and roots were significantly higher for high *gdhA*-expressors than control line in both high and low N conditions (Fig. 2-8). The nitrogen contents remaining in the Yoshida nutrient solution after the treatment for the three transgenic lines were significantly lower than that for control line in both high and low N conditions, indicating that the transgenic lines took up more nitrogen than did the control line (Fig. 2-9).

2-3-3. Expression of GS and GOGAT gene family members in leaf of control and transgenic line

The result of RT-PCR showed than the *gdhA* was successfully introduced and expressed in transgenic rice plants. The expression level of *gdhA* of TG 7 and TG 10 were higher than TG 7 (Fig. 2-10). We also examined the expression level of GS, GOGAT and GDH gene family members in leaf of the control and transgenic lines under high N condition. The Os*Gln1;1* and Os*Gln1;2* mainly functions in roots, and Os*Gln2* and Os*Glu1* are preferentially expressed in leaves (Lam et al. 1996). The expression level of Os*GDH* gene of TG 7 and TG 10 were higher than TG 5. The expression level of Os*Glu1* and Os*Gln2* gene of TG 5 and TG 10 were higher than control line, however, TG 7 was lower than control line; and Os*Gln1;1* and Os*Gln1;2* of all transgenic lines were lower than control line (Fig. 2-11).

Table 2-1. Composition of Yoshida nutrient solutions.

Element	Reagent	Preparation (g 10 liters of distilled water^{-1})
N	NH_4NO_3	914
P	$NaH_2PO_4 \cdot 2H_2O$	403
K	K_2SO_4	714
Ca	$CaCl_2$	886
Mg	$MgSO_4 \cdot 7H_2O$	3240
Mn	$MnCl_2 \cdot 4H_2O$	15.0
Mo	$(NH_4)_6 \cdot MO_7O_{24} \cdot 4H_4O$	0.74
B	H_3BO_3	9.34
Zn	$ZnSO_4 \cdot 7H_2O$	0.35
Cu	$CuSO_4 \cdot 5H_2O$	0.31
Fe	$FeCl_3 \cdot 6H_2O$	77.0
	Citric acid (monohydrate)	119

CT TG5 TG7 TG10

Fig. 2-1. Hydroponic experiment of Momiroman for nitrogen uptake efficiency. The control (CT) and transgenic (TG5, TG7, TG10) lines were treatment under high N condition at seedling stage.

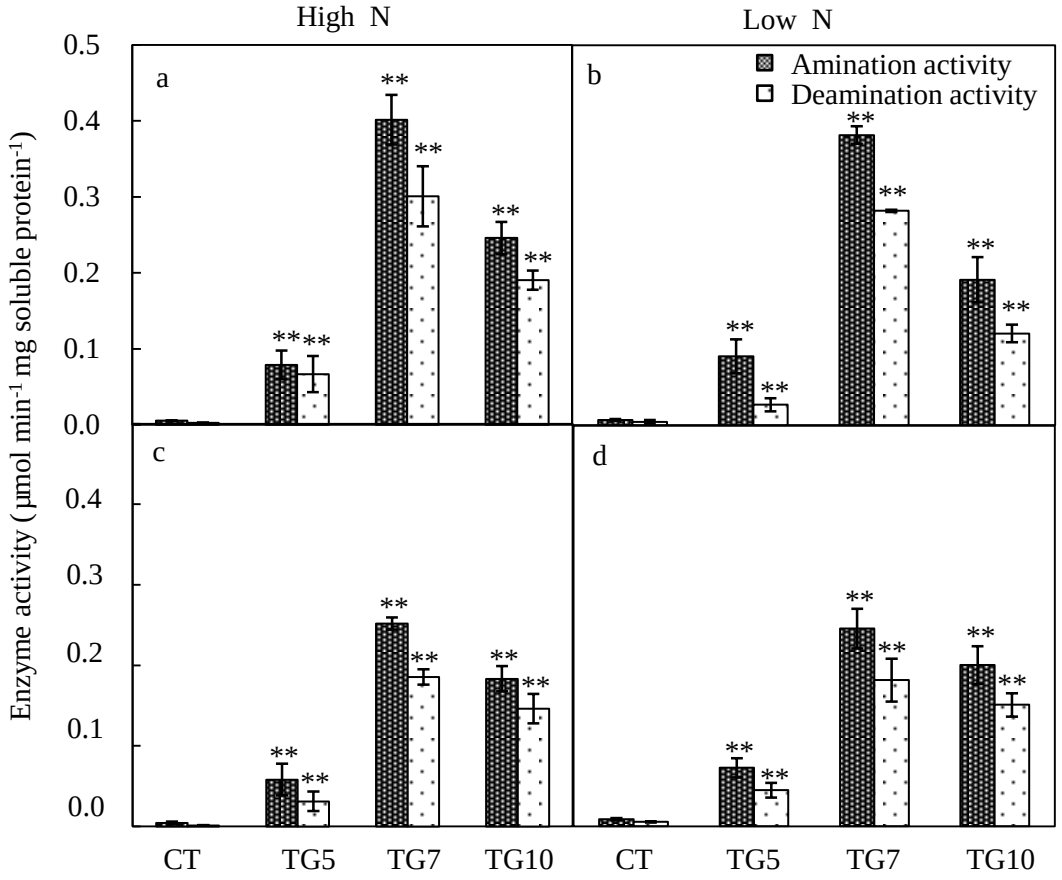
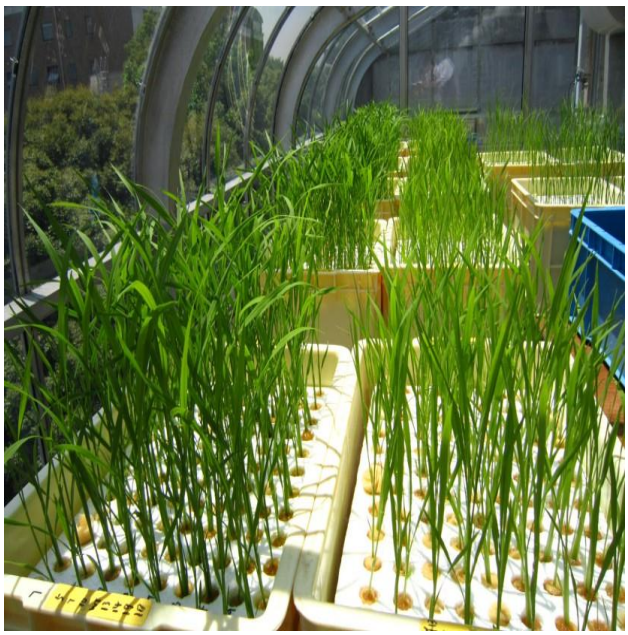


Fig. 2-2. NADP(H)-GDH activities of Momiroman. Aminating (black bars) and deaminating (white bars) activities of NADP(H)-GDH in the shoot (a, b) and root (c, d) were measured in control (CT) and transgenic (TG5, TG7 and TG10) lines under high and low N conditions after 24 d treatment. Data represent the mean values ± SD (n = 3). 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 $p < 0.01$ (**).



High N Low N

Fig. 2-3. Photographs of the seedlings after 7 d high N and low N treatment.

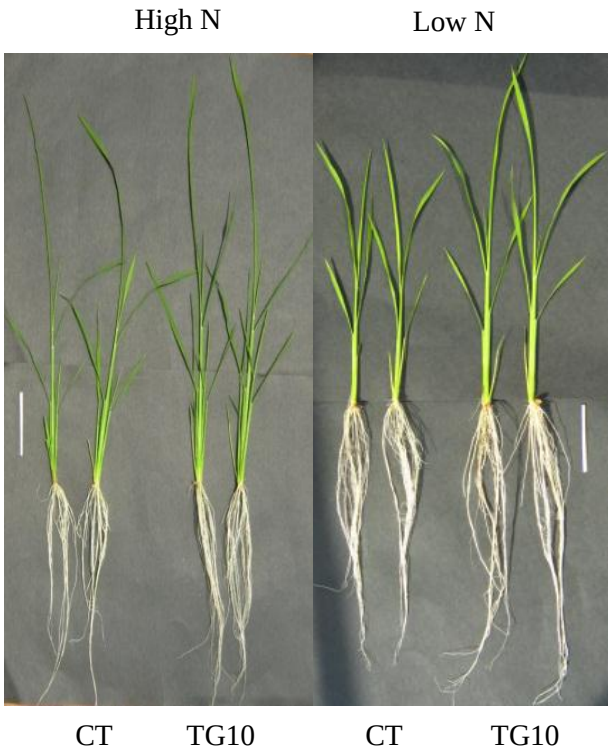


Fig. 2-4. Phenotype of control (CT) and transgenic (TG10) lines of Momiroman under high and low N conditions after 10 d treatment. Bars = 5cm.

Table 2-2. The growth characteristics of control (CT) and transgenic (TG5, TG7 and TG10) lines of Momiroman after 24 d nitrogen treatment.

Treatment	Line	Plant height (cm)	Root length (cm)
High N	CT	47.09±0.58	33.83±0.42
	TG5	47.47±0.67	34.43±0.26
	TG7	50.64±0.56 **	35.23±0.67
	TG10	48.96±1.09*	33.48±0.77
Low N	CT	33.94±0.57	37.91±0.80
	TG5	34.49±0.56	33.26±0.84 **
	TG7	39.29±0.65 **	37.84±0.89
	TG10	40.11±1.21 **	38.50±1.24

Data represent the mean values ± 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 $p < 0.01$ (**).

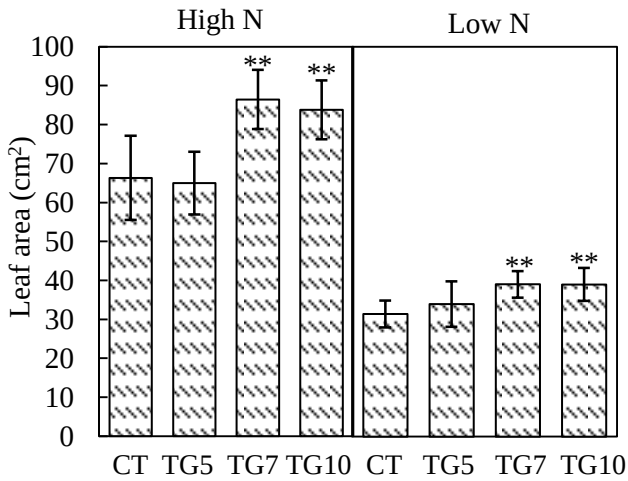


Fig. 2-5. Leaf area of control (CT) and transgenic (TG5, TG7 and TG10) lines of Momiroman under high and low N conditions at the seedling stage. Data represent the mean values ± SD (n = 9). Statistical analysis of the data was performed by one-way ANOVA. Asterisks indicate that the mean values of TG7 and TG10 lines are significantly different from that of CT at $p < 0.05$ (*) and $p < 0.01$ (**).

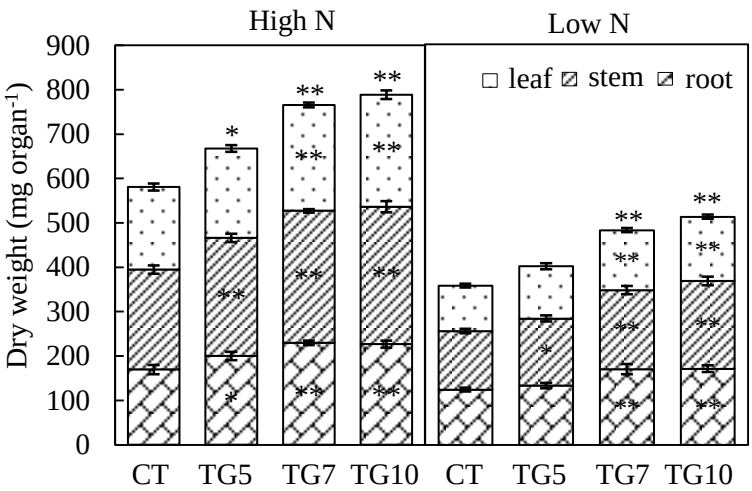


Fig. 2-6. Dry weight of control (CT) and transgenic (TG5, TG7 and TG10) lines of Momiroman. Data represent the mean values ± SD (n = 9). Statistical analysis of the data was performed by one-way ANOVA. Asterisks indicate that the mean values of TG5, TG7 and TG10 lines are significantly different from that of CT at $p < 0.05$ (*) and $p < 0.01$ (**)

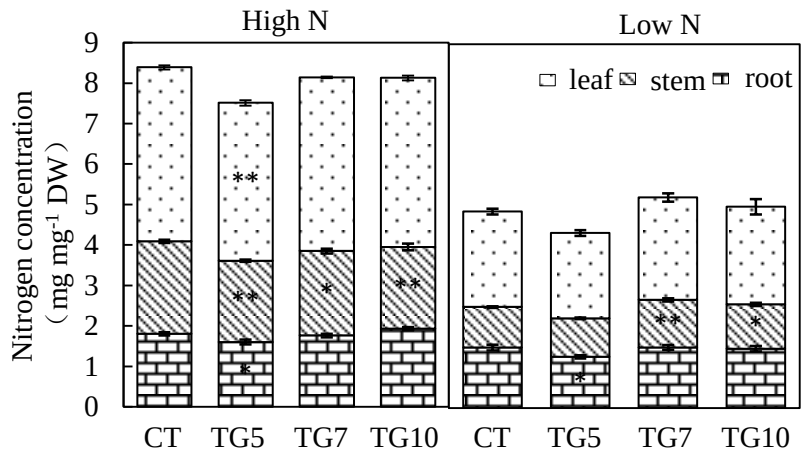


Fig. 2-7. Nitrogen concentration of control (CT) and transgenic (TG5, TG7 and TG10) lines of Momiroman under high and low N conditions at the seedling stage. Data represent the mean values ± SD (n = 4). Statistical analysis of the data was performed by one-way ANOVA. Asterisks indicate that the mean values of TG5, TG7 and TG10 lines are significantly different from that of CT at $p < 0.05$ (*) and $p < 0.01$ (**).

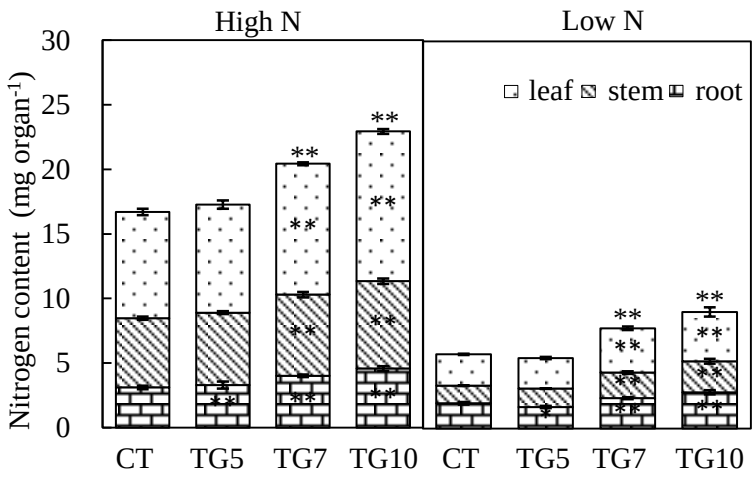


Fig. 2-8. Nitrogen contents of control (CT) and transgenic (TG5, TG7 and TG10) lines of Momiroman under high and low N conditions at the seedling stage. Data represent the mean values ± SD (n = 4). Statistical analysis of the data was performed by one-way ANOVA. Asterisks indicate that the mean values of TG5, TG7 and TG10 lines are significantly different from that of CT at $p < 0.05$ (*) and $p < 0.01$ (**).

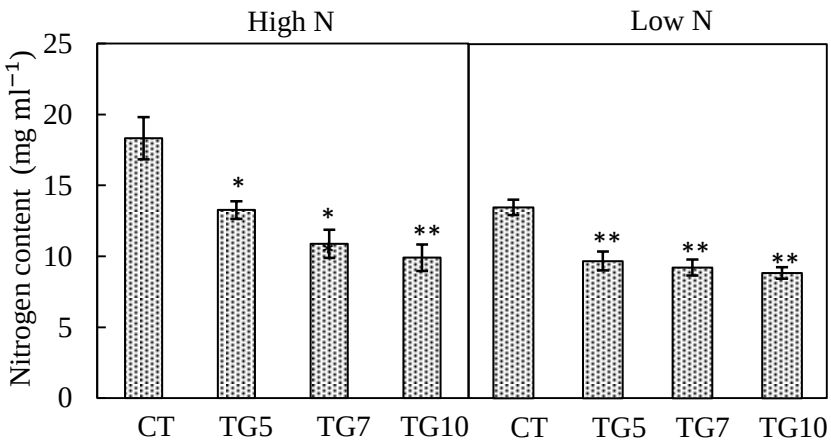


Fig. 2-9. Nitrogen contents of control (CT) and transgenic (TG5, TG7 and TG10) lines of Momiroman remained in the culture solution after 4 h high and low N treatment at the seedling stage. Data represent the mean values ± SE (n = 5). Statistical analysis of the data was performed by one-way ANOVA. Asterisks indicate that the mean values of TG5, TG7 and TG10 lines are significantly different from that of CT at $p < 0.05$ (*) and $p < 0.01$ (**).

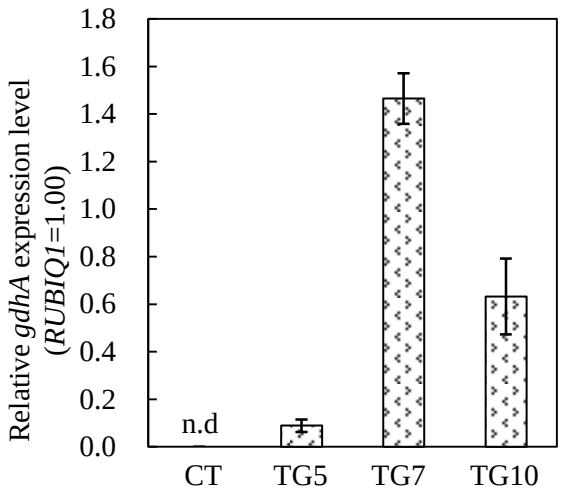


Fig. 2-10. Quantitative RT-PCR analysis of *gdhA* transcripts in the leaves of Momiroman. RT-PCR was performed with RNA from the leaves of the T_3 generations of control (CT) and transgenic (TG5, TG7 and TG10) lines. Values are expressed as the means ± SD of eight replicates, after normalization with internal control (*RUBIQ1*). The letters 'n.d.' indicates 'not detected'.

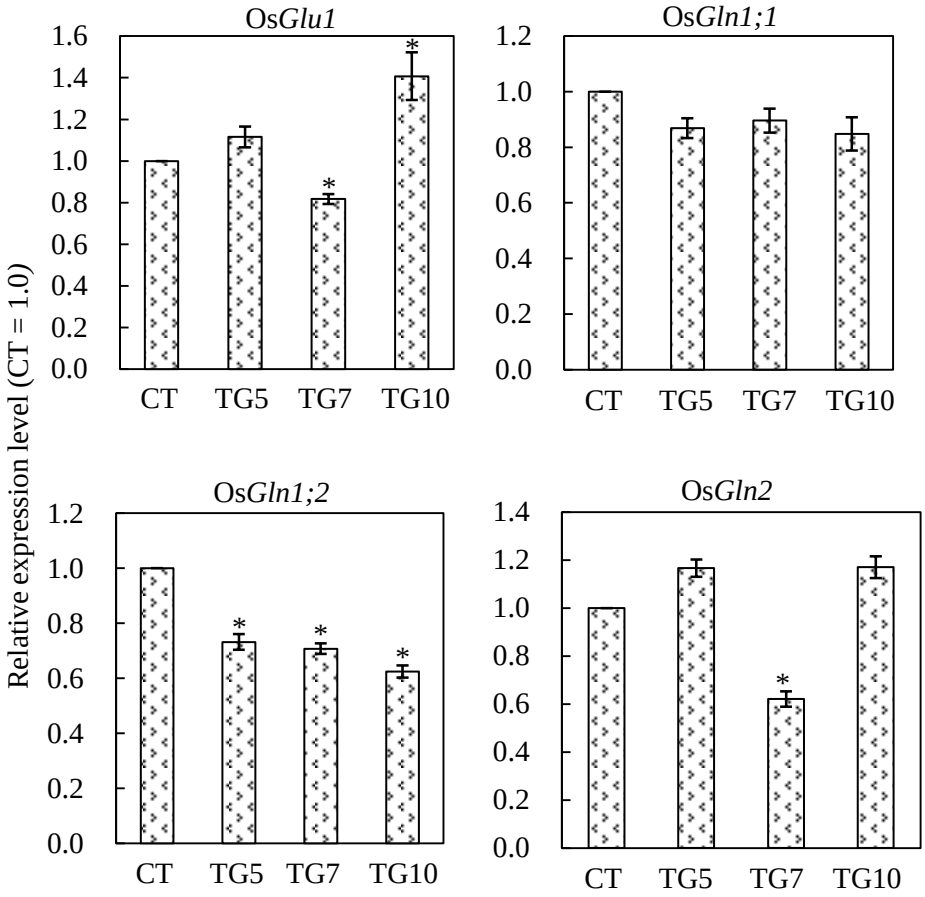


Fig. 2-11. Quantitative RT-PCR analysis of Os*Glu1*, Os*Gln1:1*, Os*Gln1:2* and Os*Gln2* in the top leaves of control (CT) and transgenic (TG5, TG7 and TG10) lines of Momiroman after 24 d nitrogen treatment. Values are expressed as the means ± SD of eight replicates, after normalization with internal control gene (*RUBIQ1*). 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 $p < 0.05$ (*).

2-4. Discussion

In this study, introduction of *gdhA* led to a significant increase in the NADP(H)-GDH activity in transgenic lines, especially with 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 both nitrogen treatment conditions (Fig. 2-2). 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 bacterial (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 rice plants may improve 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 *Ni*GDH of *Neurospora intermedia* (Wang and Tian, 2001) and *gdhA* of *A. nidulans* (Kisaka and Kida, 2003) – these transgenic plants showed high nitrogen utilization efficiency. Abiko et al., 2010 reported expression of a *gdhA* isolated from *A. niger* in transgenic rice plants and that the dry weight and nitrogen content were elevated only under high N conditions (500 μM). The present study showed that the dry weight and nitrogen content of high *gdhA*-expressors were significantly higher than control line under both high (360 μM) and low (90 μM) nitrogen conditions at seedling stage (Figs. 2-6 and 2-8).

Chapter three: Effect of *gdhA* expression on the Pn, grain weight, nitrogen content and uptake efficiency at the harvest stage

3-1. Introduction

It was shown in the previous chapter that the introduction of a fungal *gdhA* into forage rice could lead to better growth, higher dry weight and nitrogen content by enhancing nitrogen uptake efficiency under both high N and low N conditions at seedling stage. 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 *Pc*GDH transgenic plants underwent no apparent changes, yet the 1000-grain weights and panicle numbers were augmented significantly compared with control plants. In this chapter, to assess the effects of the *gdhA* introduction on the productivity of forage rice, the control and transgenic rice plants were grown under hydroponics culture conditions, the Pn, carbon and nitrogen content, grain weight and nitrogen uptake efficiency were examined under both high N and low N treatment conditions.

3-2. Materials and Methods

3-2-1. Plant materials and growth

Seeds of transgenic (T_2) and control rice plants were sown on 10 May, and grown hydroponically with Yoshida nutrient solution in a greenhouse (day/night cycle of 14/10 h

and 25/20℃). The seedlings were grown under high or low N treatment conditions respectively. The photosynthetic rate (Pn) of control and transgenic line was measured at heading stage and the nitrogen and carbon contents were measured at harvest stage.

3-2-2. Measurement of photosynthetic rates

Pn 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℃ and 75% relative humidity, with the cuvette flow constant at 300 mL min^{-1}. To obtain a light response curve, the leaf chamber CO_2 concentration was maintained at 390 μmol mol^{-1}, and Pn was measured at the following photosynthetic photon flux densities: 2000, 1500, 1000, 500, 300, 200, 100, 50, 20 and 0 μmol m^{-2} s^{-1}.

3-2-3. Measurement of nitrogen and carbon contents

Shoots and roots of control and transgenic lines were harvested and dried at 80℃ for 3 d. Dried samples were weighed with a microbalance and ground with Multi-Beads Shocker (Yasui-Kikai, Osaka, Japan). The nitrogen and carbon contents of samples and grains were measured using an NC analyzer (vario MAX CN model; Elementar, Hanau, Germany) according to the manufacturer's instructions.

3-3. Results

3-3-1. 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 evaluate this point, the light responses of Pn were measured

for both control and transgenic 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 (Fig. 3-1).

3-3-2. Effect of *gdhA* expression on carbon and nitrogen content and nitrogen concentration at the harvest stage

The nitrogen concentrations of transgenic lines did not differ from control line (Fig. 3-2). In both high and low N plants, the nitrogen and carbon 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*-expressor at the harvest stage (Figs. 3-3 and 3-4). The tiller numbers of high *gdhA*-expressors were higher than control line, while the low *gdhA*-expressor significantly lower than those of control line under both high and low N conditions (Fig. 3-5).

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 TG 7, 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 TG 5 lines (Table 3-1).

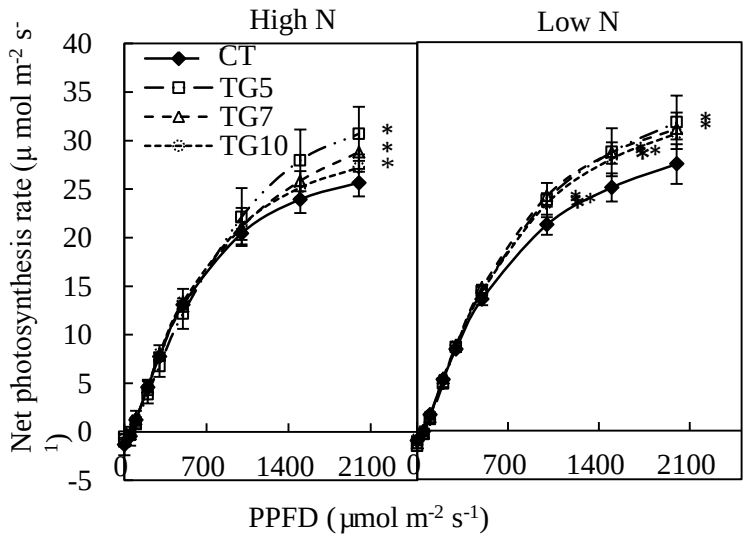


Fig. 3-1. The light response curves of Pn of control (CT) and transgenic (TG5, TG7 and TG10) lines of Momiroman under high and low N conditions at the heading stage. Data represent the mean values ± SD (n = 4). Statistical analysis of the data was performed by one-way ANOVA. Asterisks indicate that the mean values of TG5, TG7 and TG10 lines are significantly different from that of CT at $p < 0.05$ (*) and $p < 0.01$ (**)

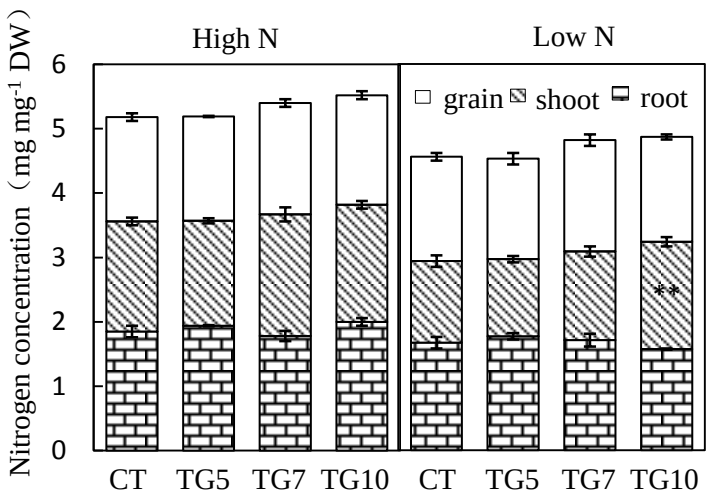


Fig. 3-2. Nitrogen concentration of control (CT) and transgenic (TG5, TG7 and TG10) lines of Momiroman under high and low N conditions at the harvest stage. Data represent the mean values ± SD (n = 4). Statistical analysis of the data was performed by one-way ANOVA. Asterisks indicate that the mean values of TG10 line is significantly different from that of CT at $p < 0.01$ (**).

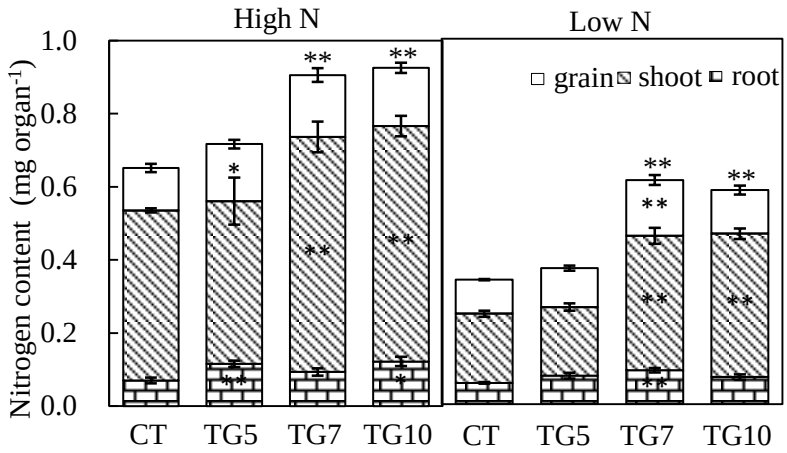


Fig. 3-3. Nitrogen contents of control (CT) and transgenic (TG5, TG7 and TG10) lines of Momiroman under high and low N conditions at the harvest stage. Data represent the mean values ± SD (n = 4). Statistical analysis of the data was performed by one-way ANOVA. Asterisks indicate that the mean values of TG5, TG7 and TG10 lines are significantly different from that of CT at $p < 0.05$ (*) and $p < 0.01$ (**).

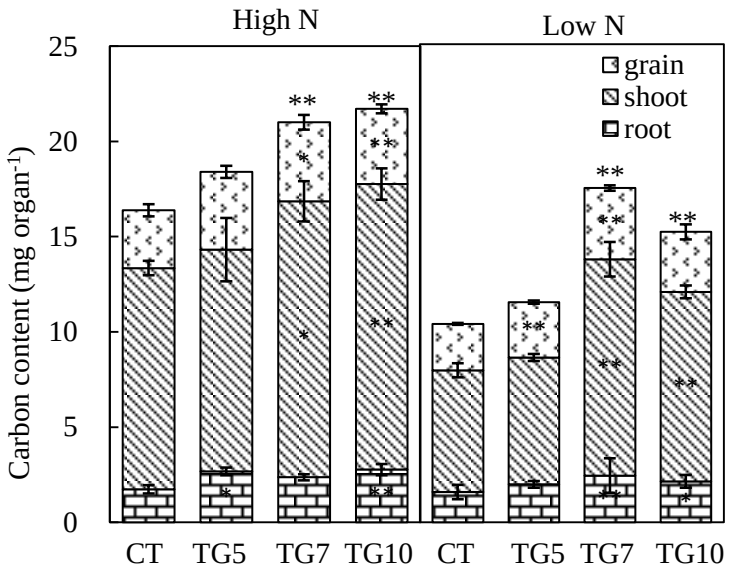


Fig. 3-4. Carbon content of control (CT) and transgenic (TG5, TG7 and TG10) lines of Momiroman were measured. Data represent the mean values ± SD (n = 4). Statistical analysis of the data was performed by one-way ANOVA. Asterisks indicate that the mean values of TG5, TG7 and TG10 lines are significantly different from that of CT at $p < 0.05$ (*) and $p < 0.01$ (**).

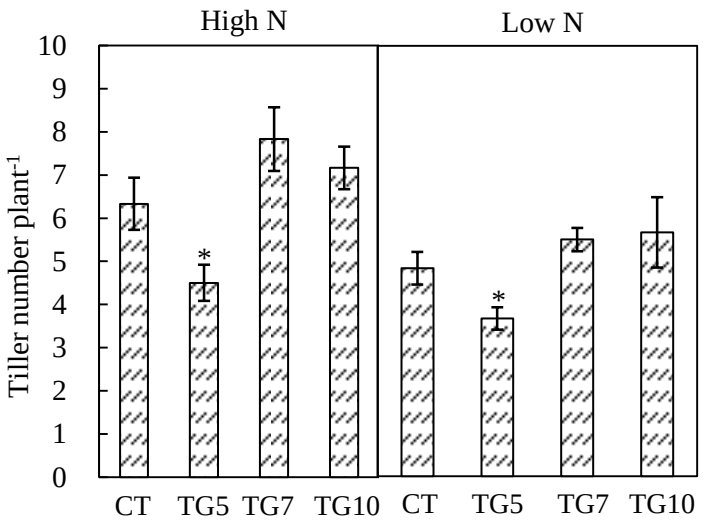


Fig. 3-5. The tiller number of control (CT) and transgenic (TG5, TG7 and TG10) lines of Momiroman at the harvest stage. Data represent the mean values ±SD (n = 6). Statistical analysis of the data was performed by one-way ANOVA. Asterisks indicate that the mean values of TG5 line is significantly different from that of CT at $p < 0.05$ (*) .

Table 3-1. The agronomic traits and nitrogen uptake efficiency of control (CT) and transgenic (TG5, TG7 and TG10) lines of Momiroman under high and low N conditions.

Treatment	Line	Panicle number ($hill^{-1}$)	Spikelet number ($plant^{-1}$)	1000-grain weight (g)	Grain weight per hill (g $plant^{-1}$)	Sink capacity (g $plant^{-1}$)	Nitrogen uptake efficiency (%)
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 **

[1]Nitrogen uptake efficiency=nitrogen content in plant / total amount of nitrogen supplied in Yoshida nutrient solution. 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 $p < 0.01$ (**)

3-4. Discussion

Nitrogen fertilizer strongly affects plant growth and dry matter productivity. It has been found that photosynthesis is strongly influenced by the nutrient status, especially the nitrogen level, of cereal crops. Nitrogen can influence photosynthesis (hence growth) through affecting leaf area itself or through changes in rate per unit leaf (Shen and Liao 1985). The higher nitrogen content of leaves was due to the greater accumulation of nitrogen in the plant before heading and during the ripening stage and the more effective partitioning of nitrogen to leaves during the ripening stage, which resulted in the maintenance of a high rate of photosynthesis during ripening (Ookawa et al., 2003). 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, the leaf area and nitrogen content of high *gdhA*-expressors were significantly higher than control line under both nitrogen treatment conditions, resulting in higher Pn in comparison with control (Fig. 3-1). These results suggest that transgenic lines had an enhanced capability for photosynthesis.

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 TG 7, similar enhancement effects were also observed under the low N conditions.

Chapter four: Effect of *gdhA* expression on the abiotic stress

4-1. Introduction

The most important cereal crop in the world is rice, yielding one third of the total carbohydrate source. Three billion people consider rice as their stable food, accounting for 50–80% of their daily calorie intake. Rice is a salt sensitive monocot (Darwish et al., 2009). Salinity is a limiting environmental factor for plant production, and is becoming more prevalent as the intensity of agriculture increases. Around the world, 100 million ha, or 5% of arable land, is adversely affected by high salt concentrations, which reduce crop growth and yield (Gunes, et al., 2007). Salt stress causes a series of physiological and biochemical changes and affects the growth and development, finally yield and quality of plants (Boyer, 1982).

Since it has been reported that transgenic plants expressing fungal or bacterial GDH showed improved tolerance to abiotic stresses (Lightfoot et al., 2007; Du et al., 2014), in this chapter, we examined the effect of exogenously introduced NADP(H)-GDH on salt and drought tolerance capacity of rice.

4-2. Materials and Methods

4-2-1. Plant materials and growth

Seeds of transgenic and control rice plants were sown on 2 May, and grown hydroponically with Yoshida nutrient solution in a greenhouse (day/night cycle of 14/10 h and 25/20 ℃). 18-d-old seedlings were transferred to 1/4 Yoshida nutrient solution, while

the plants were subjected to salt stress by respectively adding 50, 100 and 150 mM NaCl to for 1 week; drought stress by adding 10% polyethylene glycol 6000 (PEG 6000) for two weeks; The nutrient solution was daily adjusted to pH 5.0, and the solution was exchanged every 3 d. The samples for activities of NADP(H)-GDH were immediately frozen in liquid nitrogen and kept at –80 ℃ until use. The samples for dry weight and nitrogen content were divided into shoots and roots.

4-2-2. Enzyme assays

The frozen shoots 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 ℃. 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 NH_4Cl, 10 mM 2-OG and 0.1 mM β-NADP(H) 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 β-$NADP^+$ at pH 9.3. The reaction was routinely started by the addition of coenzyme (β-NADP(H) or β-$NADP^+$, 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 concentration 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.

4-2-3. Measurement of dry weight, nitrogen content and concentration

Shoots and roots of control and transgenic lines were harvested and dried at 80℃ for 3 d. Dried samples were weighed with a microbalance and ground with Multi-Beads Shocker (Yasui-Kikai, Osaka, Japan). The nitrogen contents and concentration of samples were measured using an NC analyzer (vario MAX CN model; Elementar, Hanau, Germany) according to the manufacturer's instructions.

4-3. Results

4-3-1. Effect of *gdhA* expression on tolerance of salt stress at the seedling stage

4-3-1-1. GDH activities of transgenic rice plants expressing *gdhA*

To examine the tolerance of transgenic rice plants to salt, the seedlings of control and transgenic lines at the three-leaf stage were subjected to salt stress. 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 (Fig. 4-1). Moreover, amination activity was higher than deamination activity (Fig. 4-1).

4-3-1-2. Effect of *gdhA* expression on growth, dry weight, nitrogen concentration and content

The transgenic lines remained healthy with green leaves and stems, whereas leaves of control line exhibited chlorosis following NaCl treatment, especially for 150 mM NaCl (Fig.

4-2). The dry weights of high *gdhA*-expressors (TG 7 and TG 10) were significantly higher than control line in all NaCl treatments, and dry weights for low *gdhA*-expressor (TG 5) were also significantly higher than control line under the 150 mM NaCl treatment. Compared with control conditions, the dry weight of control line dropped to 60.3%, while the TG 5, TG 7 and TG 10 lines dropped to 63.2, 73.6 and 65.9%, respectively, with 150 mM NaCl treatment (Table 4-1). The nitrogen concentration of low *gdhA*-expressor was higher than control line with 150 mM NaCl treatment, but there were no differences between the other transgenic lines and control line regardless of NaCl treatment (Fig. 4-3). 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*-expressor than control line under 150 mM NaCl treatment (Fig. 4-4).

4-3-2. Effect of *gdhA* expression on tolerance of drought stress at the seedling stage

The transgenic lines remained healthy with green leaves, whereas leaves of control line exhibited chlorosis, especially for the first and second leaf under 10% PEG treatment (Fig. 4-5). The dry weights of high *gdhA*-expressors were significantly higher than control line under 10% PEG treatment (Fig. 4-6). The nitrogen concentrations of roots of high gdhA-expressors were significantly higher than control line, although there were no differences in the nitrogen concentration for shoots between the transgenic lines and control line under 10% PEG treatment (Fig. 4-7). However, the nitrogen content of high *gdhA*-expressors were significantly higher than control line under 10% PEG treatment (Fig. 4-8).

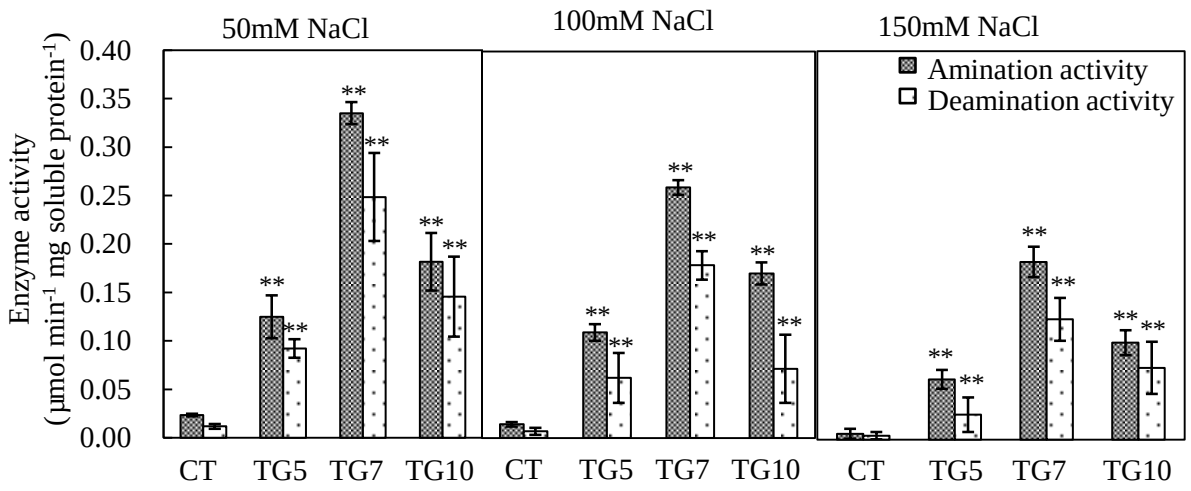


Fig. 4-1. Aminating (black bars) and deaminating (white bars) activities of NADP (H)-GDH in the shoot of control (CT) and transgenic (TG5,TG7 and TG10) lines of Momiroman were measured after 1 week treatment under different salt stress conditions. Data represent the mean values ± SD (n = 3). Statistical 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 $p < 0.01$ (**).

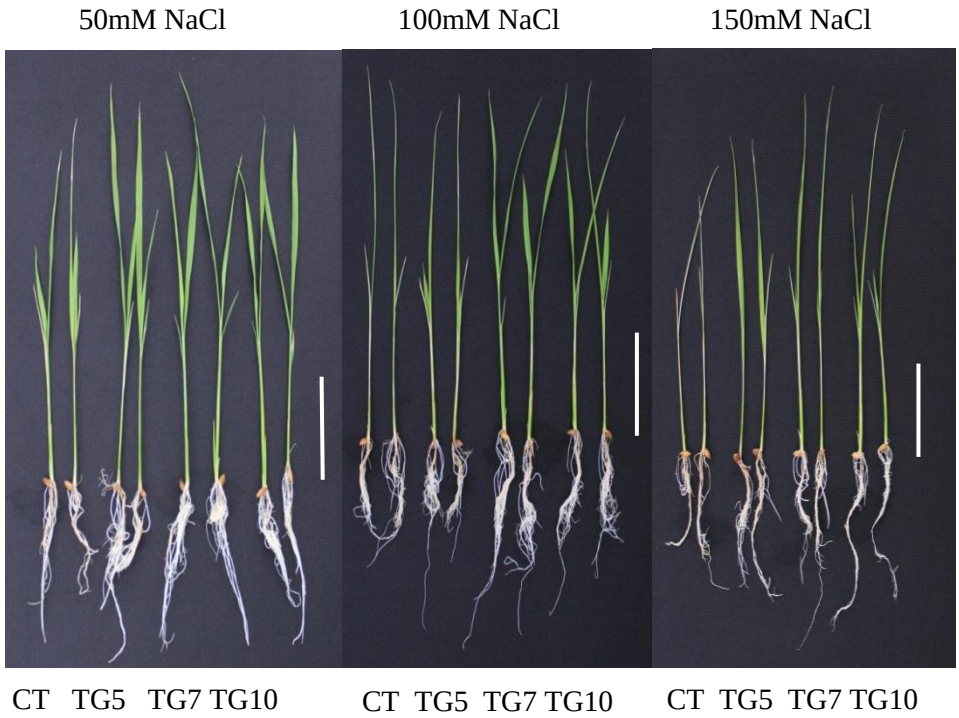


Fig. 4-2. Phenotype of control (CT) and transgenic (TG5,TG7 and TG10) lines of Momiroman after 1 week treatment under different salt stress conditions. Bars = 5 cm.

Table 4-1. Effect of different salt stress on dry weight of control (CT) and transgenic (TG5,TG7 and TG10) lines of Momiroman.

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	177.8±1.6	224.6±2.3**	203.1±1.0**
	(85.2)	(82.3)	(103)	(93.7)
100	161.5±5.4	166.6±3.3	196.9±3.8**	191.2±2.5**
	(80.4)	(77.1)	(90.3)	(88.2)
150	121.1±3.0	136.6±1.4**	160.5±1.1**	142.9±1.2**
	(60.3)	(63.2)	(73.6)	(65.9)

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 TG5, TG7 and TG10 lines are significantly different from that of CT at $p < 0.05$ (*) and $p < 0.01$ (**). Date in parentheses are percentage of treated/control.

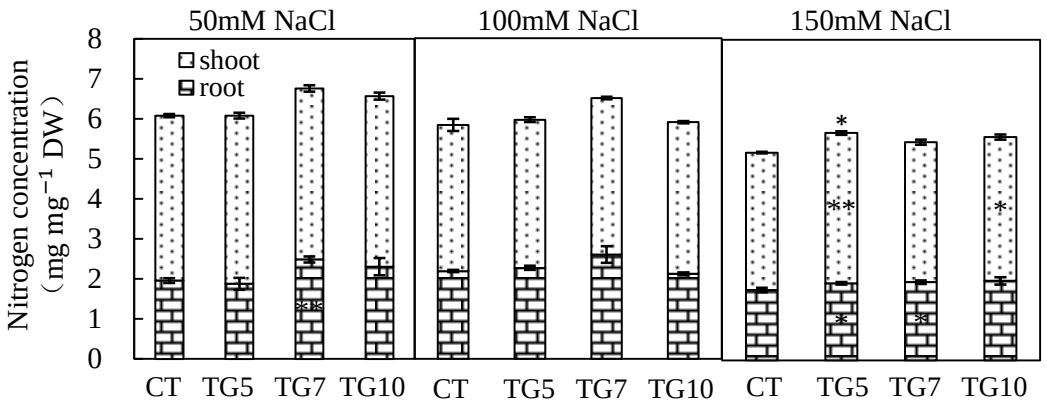


Fig. 4-3. Nitrogen concentration of control (CT) and transgenic (TG5,TG7 and TG10) lines of Momiroman under different salt stress conditions. Data represent the mean values ± SD (n = 4). Statistical analysis of the data was performed by one-way ANOVA. Asterisks indicate that the mean values of TG5, TG7 and TG10 lines are significantly different from that of CT at $p < 0.05$ (*) and $p < 0.01$ (**).

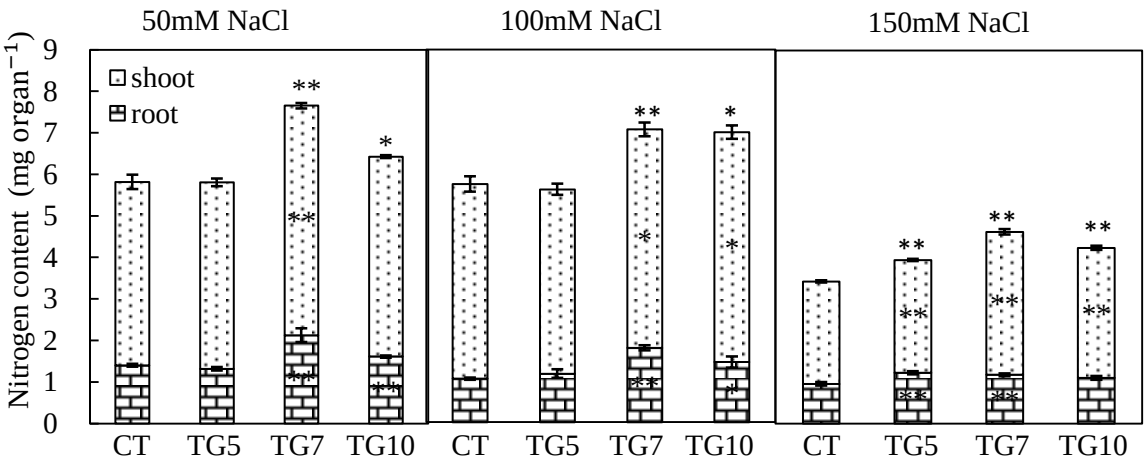


Fig. 4-4. Nitrogen contents of control (CT) and transgenic (TG5,TG7 and TG10) lines of Momiroman under different salt stress conditions. Data represent the mean values ± SD (n = 4). Statistical analysis of the data was performed by one-way ANOVA. Asterisks indicate that the mean values of TG5, TG7 and TG10 lines are significantly different from that of CT at $p < 0.05$ (*) and $p < 0.01$ (**).

Fig. 4-5. Phenotype of control (CT) and transgenic (TG5,TG7 and TG10) lines of Momiroman after 2 week treatment under 10% PEG stress condition. Bars=5cm.

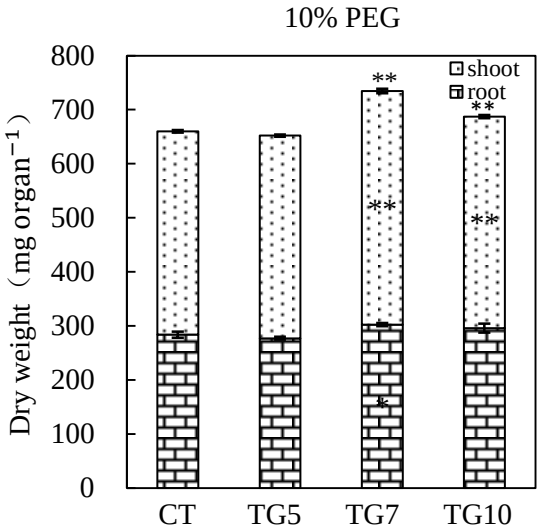


Fig. 4-6. Dry weight of control (CT) and transgenic (TG5,TG7 and TG10) lines of Momiroman under 10% PEG treatment condition. Data represent the mean values ± SD (n = 9). Statistical analysis of the data was performed by one-way ANOVA. Asterisks indicate that the mean values of TG7 and TG10 lines are significantly different from that of CT at $p < 0.05$ (*) and $p < 0.01$ (**)

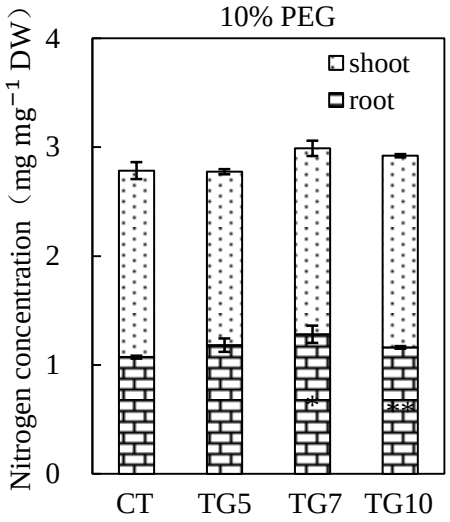


Fig. 4-7. Nitrogen concentration of control (CT) and transgenic (TG5,TG7 and TG10) lines of Momiroman under 10% PEG treatment condition. Data represent the mean values ± SD (n = 4). Statistical analysis of the data was performed by one-way ANOVA. Asterisks indicate that the mean values of TG7 and TG10 lines are significantly different from that of CT at $p < 0.05$ (*) and $p < 0.01$ (**).

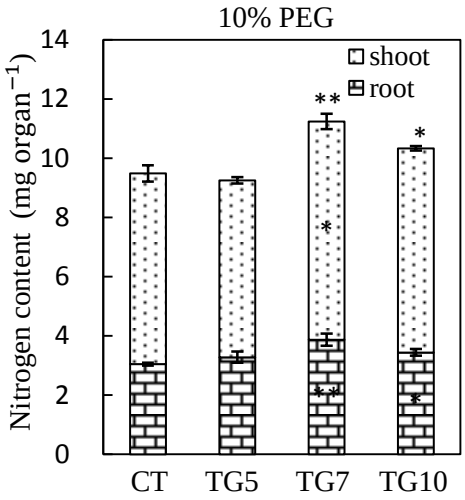


Fig. 4-8. Nitrogen content of control (CT) and transgenic (TG5,TG7 and TG10) lines of Momiroman under 10% PEG treatment condition. Data represent the mean values ± SD (n = 4). Statistical analysis of the data was performed by one-way ANOVA. Asterisks indicate that the mean values of TG7 and TG10 lines are significantly different from that of CT at $p < 0.05$ (*) and $p < 0.01$ (**).

4-4. Discussion

According to previous reports, transgenic plants expressing fungal or bacteria GDHs can show improved drought or herbicide tolerance (Ameziane et al., 2000; Nolte et al., 2004; Lightfoot et al., 2007; Du et al., 2014). Therefore, the introduction of NADP(H)-GDH from microorganisms could improve plant tolerance under stress conditions. By 2009, 35% of all paddy fields in Japan had been abandoned (MAFF, 2011). A shift from rice to alternative crops such as soybean, wheat and buckwheat 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_4^+ concentration in plant tissues such as leaves of rice or soybean increases under salt stress (Bourgeais-Chaillou et al., 1992; Lutts et al., 1999). NAD(H)-GDH activity was enhanced with increased NH_4^+ concentration, alleviating the toxic effect of NH_4^+ 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 (Fig. 4-1). 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*-expressor also showed similar enhancement under 150 mM NaCl treatment (Fig. 4-4 and Table 4-1). Our data indicated that transgenic plants

expressing fungal *gdhA* significantly enhanced their tolerance to salt stress compared to control line, suggesting that higher NADP(H)-GDH activity might decrease the NH_4^+ accumulation in tissues under salt stress.

General Discussion

The gene *gdhA* from *Escherichia coli*, that encodes a NADPH-dependent glutamate dehydrogenase (GDH), directs a novel pathway in transgenic plants that potentially allows an increase in ammonium assimilation. Compared with the GS/GOGAT pathway, GDH does not use ATP, and ammonia assimilation via the GDH pathway is therefore more energy-efficient (Windass et al., 1980; Helling, 1998). Consequently, the introduction of the exogenous GDH gene into crop plants may enhance the absorption and utilization of nitrogen fertilizer. Some transgenic crops have already been generated using the fungal NADP(H)-GDH gene, such as *NiGDH* of *Neurospora intermedia* (Wang and Tian, 2001), *gdhA* of *A. nidulans* (Kisaka and Kida, 2003), and *gdhA* of *A. niger* (Abiko et al., 2010); these transgenic plants show high nitrogen utilization efficiency.

Recently, in Japan, rice has been used for feeding livestock as well as for human food (Sittiya et al., 2011). Some new varieties for whole-crop silage such as Leafstar, Tachisuzuka and Momiroman, has been developed. In the present study, *gdhA* was introduced into forage rice (cv. Momiroman) to investigate its effects on some physiological traits such as growth, photosynthesis, nitrogen use efficiency, abiotic stress response in the transgenic rice plants. In this study, we successfully produced transgenic forage rice plants that expressed a fungal NADP(H)-GDH (*gdhA*) under the control of a constitutive EF1β promoter (Fig. 1-2). The pot experiments were performed to investigate the effect of *gdhA* in forage rice. Plant growth analysis at the seedling stage revealed that the plant height, root length and leaf area of TG 7 and TG 10 were significantly higher than those of control line in both high and low N plants (Figs. 1-5, 1-6 and 1-7). The hydroponic experiment showed that 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 both nitrogen treatment conditions (Fig. 2-2). 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).

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 μM) (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 μM) and low (90 μM) nitrogen conditions at seedling stage (Figs. 2-6 and 2-8). Although the different outcome 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 μM but we used 90 μM as the low N condition; and, second, they used NH_4^+ as the sole nitrogen source, while we used both NO_3^- and NH_4^+. Some research has shown that partial NO_3^- nutrition can improve growth and nitrogen uptake efficiency of rice (Herbert et al., 1999; Duan et al., 2007). 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 (Figs. 2-6 and 2-8). Expression of *gdhA* in transgenic rice plants results in direct

assimilation of NH_4^+ absorbed from the roots (Abiko et al., 2010), and NH_4^+ metabolism can enhance by the presence of NO_3^- (Herbert et al., 1999). Data shown in Figure 2-9 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.

Salt stress causes a series of physiological and biochemical changes and affects the growth and development, finally yield and quality of plants. In general, growth is adversely affected when seedlings are subjected to higher levels of salt stress, whereas enzyme activity responds well to the salinity level. It has been shown that the concentration of NH_4^+ in plant tissues increased under salt stress (Lutts, et al., 1999). The rice cultivars with lower salt-tolerance had higher NH_4^+ accumulation, the degree of NH_4^+ accumulation in the roots of rice cultivars differing in salinity resistance reflected the activity change of ammonia assimilation enzymes in tissues. NH_4^+ accumulation level was negatively related with the change of GS and NAD(H)-GOGAT activity but had positive correlation with NAD(H)-GDH activity (Zhou et al., 2004). In the present study, the NADP(H)-GDH activities of transgenic lines were obviously higher than control line under all NaCl treatment conditions (Fig. 4-1). The nitrogen concentration of high *gdhA*-expressors were not much different from control line, but the dry weight and nitrogen content of high *gdhA*-expressors were significantly higher than those of control line under different salt stress conditions; not only the dry weight and nitrogen content but also the nitrogen concentration of low *gdhA*-expressor also showed significantly higher than those of control line under 150 mM NaCl treatment (Figs. 4-3, 4-4 and Table 4-1). Our data indicated that transgenic plants expressing fungal *gdhA* significantly enhanced their tolerance to salt stress

compared to control line. In short, the transgenic lines with higher tolerance to salt stress maintained higher NADP(H)-GDH activity, which played an important role in decreasing the NH_4^+ accumulation in tissues under salt stress.

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 (Egami et al. 2012). In our study, we found that the chlorophyll , relative RuBisCO , and soluble protein concentration in the transgenic rice plants (TG 7 and TG 10) was higher than that in the controls regardless of the nitrogen conditions (Figs. 1-9,1-10 and 1-11). And the leaf areas were markedly larger for the high *gdhA*-expressors than control line (Figs. 1-7 and 2-5). 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 (Fig. 3-1). These results suggest that transgenic lines had an enhanced capability for photosynthesis. Some studies have shown that the introduction of a NADP(H)-dependent GDH into tobacco or rice led to better growth and yields (Ameziane et al., 2000; Abiko et al., 2010). 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.

Summary

The excessive application of nitrogen fertilizer to maximize crop yields causes negative environment effects such as pollution and ecological imbalance. Therefore, there is a need to improve the ability of crop plants to utilize nitrogenous fertilizers by promoting nitrogen uptake, assimilation and metabolism. Recently, rice has been used for feeding livestock as well as for human food. In Japan, forage rice, a new variety for whole-crop silage has been developed. The present thesis shows successful generation of transgenic rice plants expressing *gdhA*, and examines the effects of the introduction of a fungal glutamate dehydrogenase gene (*gdhA*) into forage rice by analyzing the transgenic rice plants in terms of growth, source function, dry matter production, nitrogen contents and nitrogen uptake efficiency.

Chapter one: Generation of transgenic rice plants expressing *gdhA* and variety selection

To express introduced *gdhA* constitutively within transgenic rice plants, we used a rice elongation factor-1 beta promoter (EF1β; Gene Locus Os04g0118400) and a rice prolamin 10 terminator (P10; Os03g0766000). The resultant EF1βpromoter–*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 results of PCR and RT-PCR indicated that *gdhA* was successfully introduced and expressed in the transgenic plants. By the results of pot experiment, introduction of *gdhA* led to a significant increase in the plant height, root length, leaf area, the chlorophyll concentration, relative RuBisCO

concentration and soluble protein concentration in two of transgenic lines (TG 7 and TG 10) of Momiroman under both high and low nitrogen treatment conditions. The leaf, stem and root dry weights of transgenic line (TG 30) of Yamahoshi were higher than those of control line in both high and low N plants. Because of all the results of transgenic plants of Leafstar were lower than control line and the poor qualities of the seeds, and previously our research group already reported expression of a *gdhA* in food rice plants (cv. Yamahoshi) (Abiko et al., 2010). So we decided to continue the following research with Momiroman.

Chapter two: Effect of *gdhA* expression on GDH activities, dry weight, nitrogen content and nitrogen uptake efficiency at the seedling stage

In order to reduce the influence of factors or substances other than that of nitrogen, hydroponic experiments (NH_4NO_3 as nitrogen source) were carried out from this chapter. We used 360 μM and 90μM nitrogen as high nitrogen (high N) and low nitrogen (low N) treatment. All the transgenic plants used for analyses were checked by a genomic PCR with regard to the introduction of *gdhA.* The 6-week-old seedlings were sampled after 10 and 24 d of nitrogen supplementation. The GDH activities were higher in TG 7 and TG 10 than in TG5 regardless of nitrogen conditions (Fig. 2-2). We called the former two 'high *gdhA*-expressors' and the latter 'low *gdhA*-expressor'. 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 in both high and low nitrogen conditions. These results suggested that the source ability was enhanced by the *gdhA* introduction. The nitrogen content and nitrogen uptake efficiency of high *gdhA*-expressors were significantly higher than control line under both high and low N treatment conditions.

Chapter three: Effect of *gdhA* expression on the Pn, grain weight, nitrogen content and uptake efficiency at the harvest stage

According to some previous reports, transgenic plants expressing fungal or bacterial GDHs that grown in the field produced significantly higher grain weight than did control plants (Ameziane et al., 2000; Abiko et al., 2010; Zhou et al. 2014). To assess the effects of the *gdhA* introduction on the photosynthetic capacity and productivity of forage rice, the control and transgenic rice plants were grown under hydroponics culture conditions. Experiments in chapter 2 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 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.

Chapter four: Effect of *gdhA* expression on the abiotic stress

It has been reported that transgenic plants expressing fungal or bacterial GDH showed improved tolerance to abiotic stresses (Nolte et al., 2004; Lightfoot et al., 2007; Du et al., 2014). Therefore, the introduction of NADP(H)-GDH from microorganisms could improve plant tolerance under stress conditions. To examine the effect of exogenously introduced NADP(H)-GDH on salt and drought stress, we examine the salt and drought tolerance capacity of the transgenic and control plants at the three-leaf stage under 50mM, 100mM, 150mM NaCl and 10% PEG 6000 solution. The NADP(H)-GDH activities were

significantly higher for transgenic than control plants under high salt conditions. The dry weights and nitrogen content of high *gdhA*-expressors were significantly higher than control line under all NaCl concentration and 10% PEG treatments conditions.

In conclusion, the present thesis 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. In addition, *gdhA* expression in forage rice significantly enhanced their tolerance to salt and drought stress compared to control plants.

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