Tos17 insertion in NADH-dependent glutamate synthase genes leads to an increase in grain protein content in rice

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Tos17 insertion in *NADH-dependent glutamate synthase* genes leads to an increase in grain protein content in rice

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Nutrition from cereal grains sustains human life, and the protein content of rice grains is an important source for humans. Recent forward genetics studies pointed out the significant contribution of NADH-dependent glutamate synthase (NADH-GOGAT) to increased grain protein content in durum wheat. The aim of this work was to investigate the contribution of the two NADH-GOGAT enzymes in rice, NADH-GOGAT1, and NADH-GOGAT2, to rice grain protein content, using a reverse genetics approach. The grain protein content and free amino acid concentration were determined in Tos17 insertion mutants of the structural gene of each protein. We found a significant increase in protein content in the *NADH-GOGAT2* mutant and an increase in free amino acid concentration in both the *NADH-GOGAT1* and the *NADH-GOGAT2* mutants. These results provide the first nutritional characterization of the *NADH-GOGAT* mutants. Rice containing increased protein and amino acid concentrations may have potential to contribute to improved rice nutrition. Since previous field analysis had indicated a 40% yield reduction in each of the mutants, future work should focus on improving the yield of the mutants.

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Introduction

Improving the nutritional value of cereal grains can contribute significantly to human health. Rice (*Oryza sativa* L.), bread wheat (*Triticum aestivum* L.) and maize (*Zea mays* L.) are the most important (in terms of the human diet) cereals in the world, and cereal grain protein is an important dietary source of protein, in terms both of quantity and quality (e.g. biological value), for humans (Peng et al. 2014). Although the protein content of rice is the lowest of the major cereals (Peng et al. 2014), its biological value, in terms of total N digestibility and amino acid balance, is high (Peng et al. 2014).

The metabolism of glutamine and glutamate influences the grain protein content and free amino acid composition in plants (Gadaleta et al. 2011; Nigro et al. 2013; Nigro et al. 2017). Glutamine synthetase (GS) catalyzes the condensation of glutamate and ammonium to form glutamine (Konishi et al. 2017, 2018), while glutamate synthase (GOGAT) catalyzes the transamination of glutamine to 2-oxoglutarate to form two molecules of glutamate (Tamura et al. 2010, 2011). The reaction of GS and GOGAT is cyclical, and it is known as the GS/GOGAT cycle. Glutamate decarboxylase (GAD) catalyzes the decarboxylation of glutamate to form gamma-aminobutyric acid (GABA) (Komatsuzaki et al. 2007; Akama et al. 2009), a non-protein amino acid which has several known physiological functions as a neurotransmitter in the mammalian brain (Komatsuzaki et al. 2007), and GABA has been shown to be an effective treatment for hypertension (Komatsuzaki et al. 2007; Akama et al. 2009).

Two approaches have been used to identify the genes which influence grain protein content in cereals, namely forward genetics using QTL analysis (Ye et al. 2010; Peng et al. 2014) and reverse genetics, using mutants (She et al. 2010; Wang et al. 2011). Several lines of evidence have suggested that the GS/GOGAT genes regulate grain protein content in durum wheat. GS (Gadaleta et al. 2011) and *NADH-dependent GOGAT* (Nigro et al. 2013) genes were located in the QTL regions identified as controlling grain protein content. Regression analysis indicated a significant relationship between allelic variants of *NADH-GOGAT* genes and grain protein content (Nigro et al. 2017). Methods to increase GABA concentrations in food have been investigated, and water soaking (Saikusa et al. 1994) and gaseous treatment (Komatsuzaki et al. 2007) of rice grains were effective at increasing GABA concentration in rice grains. In addition, seed expression of a truncated *GAD2* gene increased GABA concentration in rice (Akama et al. 2009).

Although forward genetics highlighted the close link between GOGAT and grain protein content, and several genetic and cultural strategies have been shown to increase GABA concentration in grain, little attention has been paid to the evaluation of grain protein content and free amino acid composition in GOGAT mutants. The reverse genetic strategy is one of the most effective strategies for relating GOGAT to grain protein content and free amino acid composition in the grains of cereal plants.

Previous studies have reported the isolation of NADH-GOGAT-deficient mutants in rice (Tamura et al. 2010, 2011) and Arabidopsis (Kojima et al. 2014; Konishi et al. 2014). A loss of either functional NADH-GOGAT1 (Tamura et al. 2010) or NADH-GOGAT2 (Tamura et al. 2011) led to a 40% decrease in grain yield. The NADH-GOGAT1 mutant exhibited decreased panicle number and spikelet number per panicle, while the NADH-GOGAT2 mutant exhibited decreases in both spikelet number per panicle and the proportion of well- ripened grains. Neither mutant showed any significant change in 1,000-spikelet weight.

Although the growth and yield of the *NADH-GOGAT* mutants has been investigated (Tamura et al. 2010, 2011), the nutritional quality of the grains of the *NADH-GOGAT* mutants remains to be elucidated; this is the focus of

the current study. The grain protein content and free amino acid composition (including that of the non-protein amino acid, GABA) were determined in *Tos17* insertion lines for *NADH-GOGAT1* and *NADH-GOGAT2*. We demonstrate that a loss of NADH-GOGAT activity led to an increase in grain protein content and free GABA concentration in the rice grains.

Results

Tos17 insertion in *NADH-GOGAT* genes did not change the weight of the individual filled grain

Data obtained in previous studies had indicated that there was no significant difference in the weight of 1,000 filled grains between the *NADH-GOGAT* mutants and their corresponding parental control, 'Nipponbare' (Tamura et al. 2010, 2011). Previous studies had grown plants in a paddy field in Kashimadai, Miyagi, Japan in 2009. In the current study, the grains were harvested from rice plants grown in a paddy field in Amamiya, Miyagi, Japan in 2014. *NADH-GOGAT1* mutants showed significant reduction in NADH-GOGAT activity in the grain (Fig. 1A). Figure 1B shows the weight of brown rice grains from 'Nipponbare' and the *NADH-GOGAT* mutants. The grain weight of neither the *NADH-GOGAT1* mutant nor that of the *NADH-GOGAT2* mutant was significantly different from that of 'Nipponbare' (Fig. 1B). The result is consistent with the results obtained in previous studies (Tamura et al. 2010, 2011), although the seeds had been cultivated and harvested under different cultural conditions. It can be observed from Fig. 1B that reasonably consistent grains were used for further analysis.

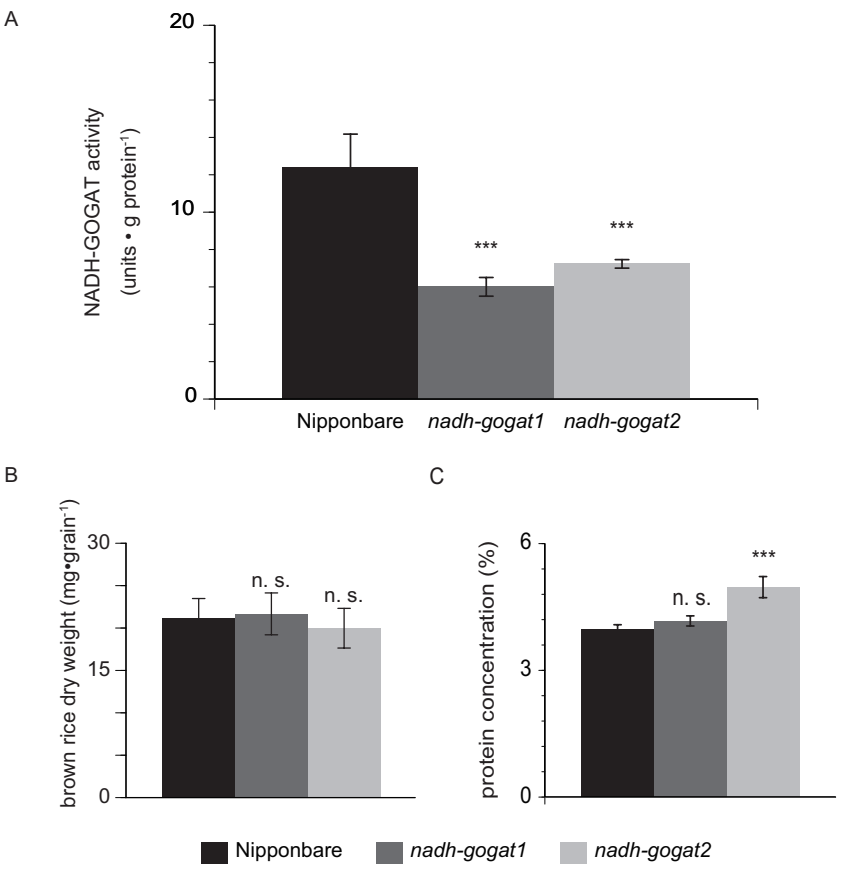

Figure 1. The weight and protein concentration of the grains of the 'Nipponbare' mutants

(A) NADH-GOGAT activity in the grains of 'Nipponbare' (black column), the *nadh-gogat1* (dark gray column) and the *nadh-gogat2* (light gray column). Data are presented as mean ± SD (n = 3). A unit of enzyme is defined as that amount that synthesizes 1 μmol glutamate per min at 30°C.

(B) The weight of the grains of 'Nipponbare' (black column), the *nadh-gogat1* (dark gray column) and the *nadh-gogat2* (light gray column). The grains were selected by soaking in NaCl solution (d = 1.06), dehusking and weighing. Bars indicate means ± standard deviation (SD) (n = 50). Significant differences between wild-type and mutants were identified by Student's t-test and are marked with asterisks: $*P<0.05$; n. s. indicates not significant.

(C) The protein concentration in the grains of 'Nipponbare' (black column), the *nadh-gogat1* (dark gray column) and the *nadh-gogat2* (light gray column). The grains were ground to a powder with a Multi-Beads Shocker. The grain powder was extracted with Tris-HCl protein extraction buffer. The protein concentration was determined with the RC DC Protein Assay Kit (Bio-Rad Laboratories Inc., Tokyo, Japan), with bovine serum albumin as a standard. Bars indicate means ± standard deviation (SD) (n = 4). Significant differences between the wild-type and the mutant were identified by Student's t-test and are marked with asterisks: $***P<0.001$; n. s. indicates not significant.

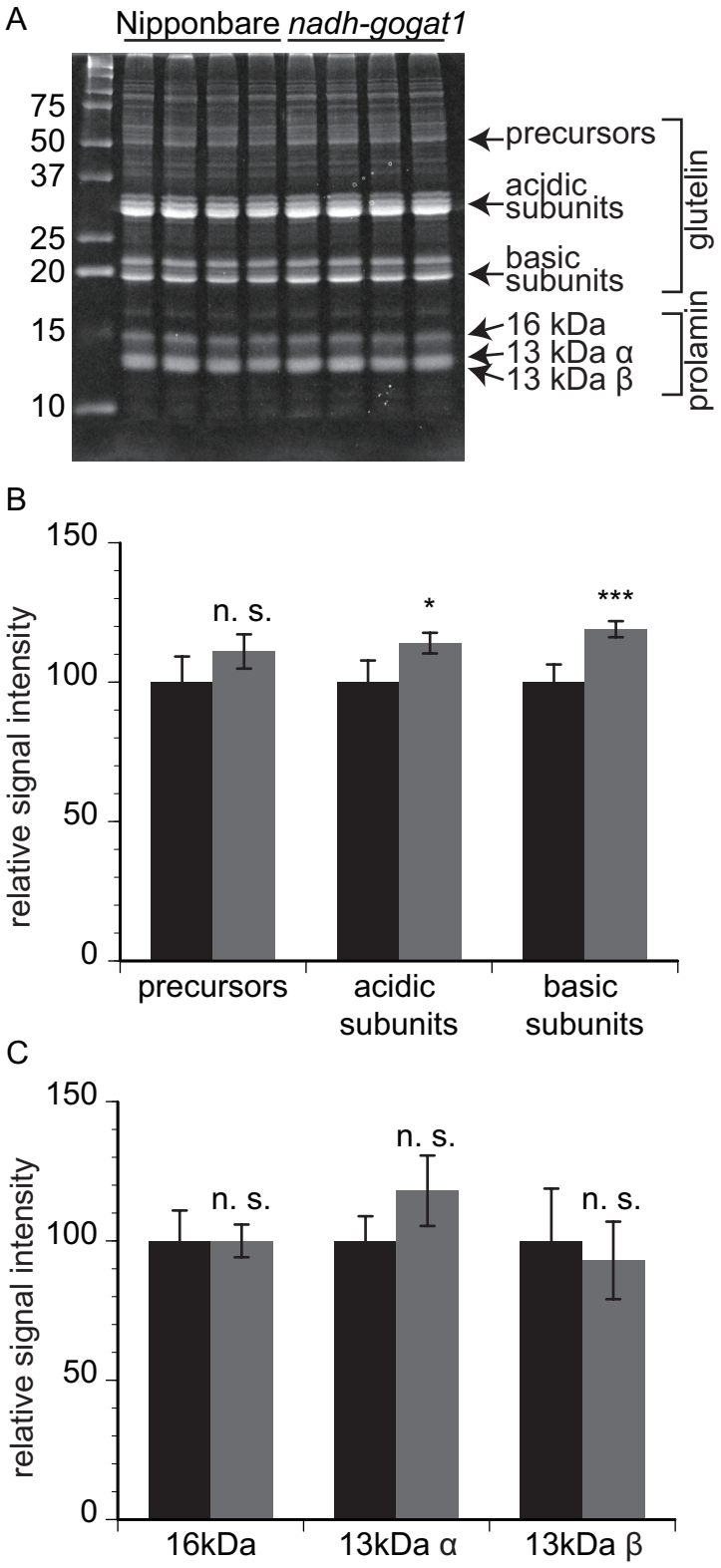

Figure 2. Protein analysis of 'Nipponbare' and *nadh-gogat1* grains

(A) SDS-PAGE analysis of total grain proteins from Nipponbare and the nadh-gogat1. Each lane contained 3.7 μL extracted total protein from one rice grain. Proteins were stained with Coomassie Blue after separation by SDS-PAGE. Arrows indicated glutelin precursors, acidic subunits, basic subunits, prolamin 16 kD, 13 kD alpha and 13 kD beta. (B) Comparison of glutelins in seeds of 'Nipponbare' (black column) and the *nadh-gogat1* (dark gray column). Glutelin precursors, acidic subunits, and basic subunits were quantified by ImageJ. (C) Composition of prolamin in seeds of 'Nipponbare' (black column) and the *nadh-gogat1* (dark gray column). Different sizes of prolamin, 16kD, 13kD alpha, and 13 kD beta, were quantified by ImageJ. Bars indicate means ± standard deviation (SD) (n = 4). Significant differences between wild-type and mutant were identified by Student's t-test and are marked with asterisks: $^{*}P<0.05$, $^{***}P<0.001$; n. s. indicates not significant.

Significant increases in seed protein concentrations were observed in the mutant grains

Since previous work (Gadaleta et al. 2011; Nigro et al. 2013; Nigro et al. 2017) had suggested a link between grain protein content and NADH-GOGAT, the concentration of protein in the grains was investigated in the *NADH-GOGAT* mutants. A loss of NADH-GOGAT2 function led to an increase of almost 20% in grain protein concentration, while the *NADH-GOGAT1* mutant did not show a significant difference in grain protein concentration, compared with 'Nipponbare' (Fig. 1C). SDS-PAGE analysis (Fig. 2A and Fig. 3A) was then conducted to investigate for qualitative differences in seed proteins between the mutant lines. Loss-of-function of NADH-GOGAT1 led to an increase in glutelin acidic (14%) and basic subunits (19%) (Fig. 2), whereas precursors of neither glutelins nor prolamins showed any marked changes in concentration, compared to those in 'Nipponbare'. On the other hand, the loss-of-function of NADH-GOGAT2 led to an increase in concentrations of both glutelins

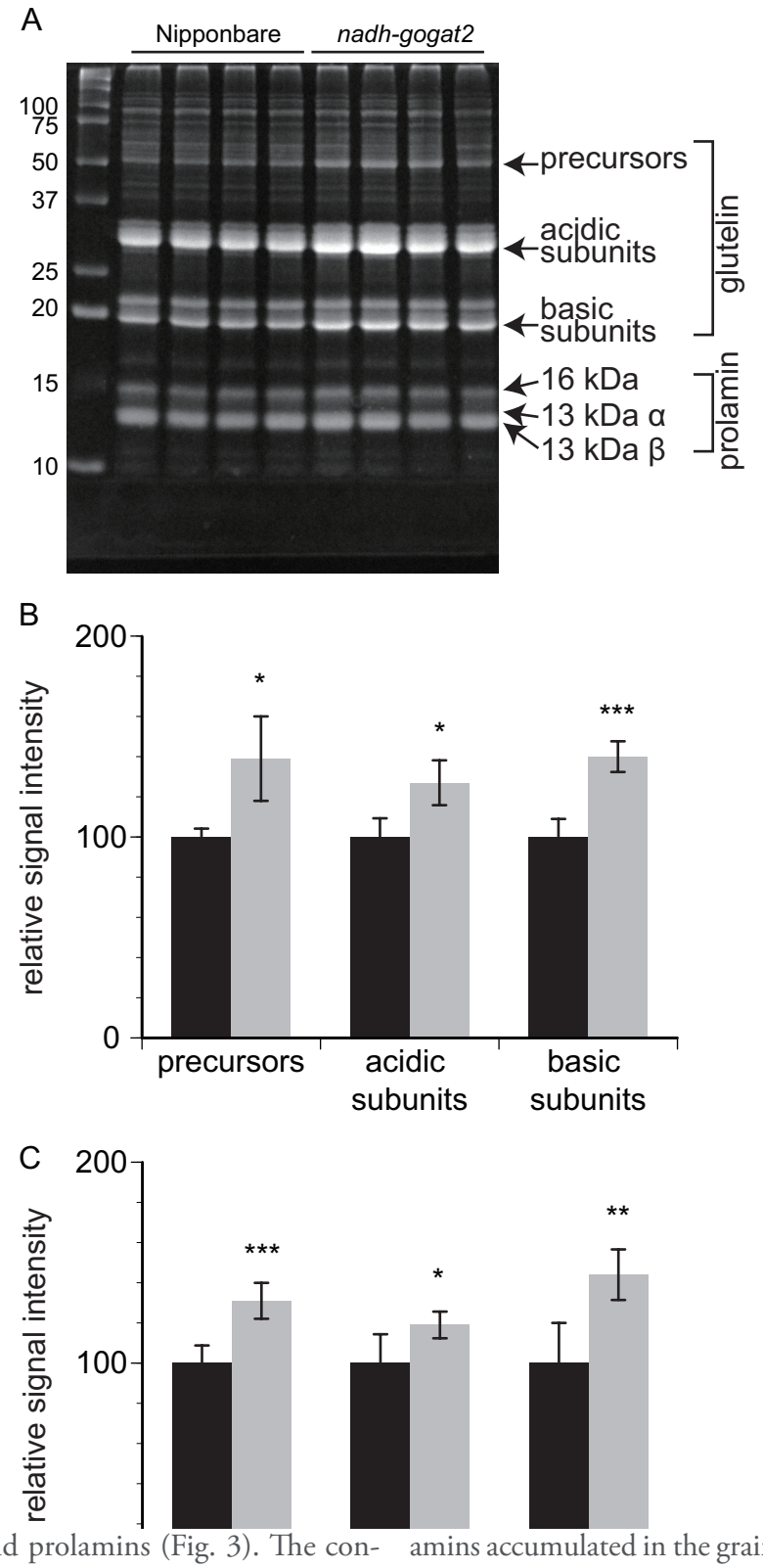

Figure 3. Protein analysis of 'Nipponbare' and *nadh-gogat2* grains

(A) SDS-PAGE analysis of total proteins from 'Nipponbare' and the nadh-gogat2. Each lane contained 3.7 μL extracted total protein from one rice grain. Proteins were stained with Coomassie Blue after separation by SDS-PAGE. Arrows indicated glutelin precursors, acidic subunits, basic subunits, prolamins 16 kDa, 13kDa alpha and 13 kDa beta. (B) Comparison of glutelins in grains of 'Nipponbare' (black column) and the *nadh-gogat2* (light gray column). Glutelin precursors, acidic subunits, and basic subunits were quantified by ImageJ. (C) Composition of prolamin in grains of 'Nipponbare' (black column) and the *nadh-gogat2* (light gray column). Different sizes of prolamin, 16kD, 13kD alpha, and 13 kD beta, were quantified by ImageJ. Bars indicate means ± standard deviation (SD) (n = 4). Significant differences between 'Nipponbare' and the mutant were identified by Student's t-test and are marked with asterisks: $*P<0.05$, $***P<0.001$; n. s. indicates not significant.

and prolamins (Fig. 3). The concentrations of all glutelins, their precursors, and their acidic and basic subunits in the *NADH-GOGAT2* mutant increased by 27–40% compared to 'Nipponbare' (Fig. 3B). Prolamins, namely the 16 kD, 13 kD alpha and 13 kD beta prolamins accumulated in the grains of the *NADH-GOGAT2* mutant to higher concentrations than in 'Nipponbare'. The concentrations of each of the prolamins in this mutant increased by 19–44% (Fig. 3C).

Free amino acid analysis in the mutant grains

Since the free amino acid composition in the grain affects the nutritional characteristics of rice, the free amino acid composition in seeds was investigated. Both the *NADH-GOGAT1* and the *NADH-GOGAT2* mutant showed higher free amino acid concentrations in their grains than did 'Nipponbare' (Table 1). The concentrations of the free amino acids in the *NADH-GOGAT* mutants increased by 48–67%, compared to 'Nipponbare' (Table 1). *Tos17* insertion in *NA-*

Table 1. Amino acid composition in the seeds of Nipponbare and NADH-GOGAT mutants

	Nipponbare		*nadh-gogat1*			*nadh-gogat2*		
His	0.07	± 0.01	0.07	± 0.01	n. s.	0.05	± 0.01	*
Asn	0.75	± 0.07	0.72	± 0.00	n. s.	0.77	± 0.18	n. s.
Ser	0.69	± 0.09	1.05	± 0.13	**	1.19	± 0.12	***
Gln	0.78	± 0.12	1.31	± 0.16	***	1.73	± 0.58	*
Arg	0.42	± 0.02	0.38	± 0.08	n. s.	0.58	± 0.03	***
Gly	0.16	± 0.01	0.26	± 0.04	***	0.28	± 0.02	***
Asp	0.40	± 0.12	0.50	± 0.26	n. s.	0.69	± 0.12	*
Glu	0.77	± 0.19	0.94	± 0.56	n. s.	1.33	± 0.33	*
Thr	0.12	± 0.01	0.21	± 0.01	***	0.20	± 0.01	***
Ala	0.40	± 0.08	0.78	± 0.30	n. s.	0.62	± 0.07	*
GABA	0.18	± 0.06	0.64	± 0.15	***	0.35	± 0.04	*
Pro	0.09	± 0.02	0.16	± 0.03	*	0.23	± 0.05	***
Lys	0.03	± 0.01	0.05	± 0.02	n. s.	0.07	± 0.01	***
Tyr	0.06	± 0.01	0.10	± 0.00	***	0.09	± 0.01	***
Val	0.18	± 0.02	0.33	± 0.07	**	0.29	± 0.03	***
Ile	0.09	± 0.02	0.15	± 0.02	*	0.13	± 0.02	*
Leu	0.06	± 0.01	0.13	± 0.02	***	0.14	± 0.01	***
Phe	0.05	± 0.00	0.08	± 0.02	*	0.09	± 0.00	***
total	5.30	± 0.70	7.87	± 1.66	*	8.83	± 1.23	***
NH_4^+	2.08	± 0.23	3.28	± 1.39	n. s.	2.51	± 0.40	n. s.

means ± SD (n = 4–5), and significant differences between Nipponbare and NADH-GOGAT mutants identified by Student's t-test are marked with asterisks: *P<0.05, **P<0.01, ***P<0.005 or n. s. indicated not significant.

DH-GOGAT led to a marked accumulation of free glutamine in the grains of the mutants (Table 1). While the concentration of free glutamate and free aspartate in the *NADH-GOGAT1* mutant did not change, the corresponding concentrations in the *NADH-GOGAT2* mutant increased by 72% and 73%, respectively, relative to 'Nipponbare' (Table 1). No significant change in free asparagine concentration was observed in either *NADH-GOGAT* mutant line (Table 1). Free GABA concentration increased almost threefold in the *NADH-GOGAT1* mutant and twofold in the *NADH-GOGAT2* mutant (Table 1). The concentration of free lysine increased (twofold) only in the *NADH-GOGAT2* mutant, while that of free threonine increased by 67–75% in both mutants (Table 1).

Discussion

In earlier studies, it was reported that a loss-of-function of either NADH-GOGAT1 or NADH-GOGAT2 led to a 40% reduction in grain yield of rice (Tamura et al. 2010, 2011), and more recent research has also suggested the importance of NADH-GOGAT in controlling grain protein content in durum wheat (Nigro et al. 2013, 2017). It is evident that the grain protein content values in rice reported here were not in close agreement with those from the forward genetic studies in durum wheat (Gadaleta et al. 2011; Nigro et al. 2013; Nigro et al. 2017). In the current work, we sought to determine whether the *NADH-GOGAT* mutants exhibited any changes in grain composition which may have implications in terms of human nutrition. The total protein concentration, protein composition, and free amino acid composition were determined in the grains of Nipponbare and the two *NADH-GOGAT* mutants. A loss-of-function mutation in NADH-GOGAT1 did not change the total protein concentration in the grains (Fig. 1C), but

the concentrations of both the acidic and basic subunits of glutelins increased by 14–19% (Fig. 2). The concentrations of free amino acids (Table 1), especially glutamine and GABA (Table 1), increased in the *NADH-GOGAT1* mutant. Whereas the *NADH-GOGAT1* mutant did not show altered grain protein concentration, the loss-of-function *NADH-GOGAT2* mutation led to an increase in grain protein concentration (Fig. 1C). The concentrations of both glutelins and prolamins increased in the *NADH-GOGAT2* mutant relative to 'Nipponbare' (Fig. 3). In addition to increased protein concentration, total free amino acid concentrations also increased in the *NADH-GOGAT2* mutant (Table 1). Glutamine and glutamate concentration increased significantly in the *NADH-GOGAT2* mutant (Table 1).

The results indicate that the contribution of NADH-GOGAT to grain number was more significant than any effect on primary ammonium assimilation or nitrogen translocation. The reverse genetics approach highlighted the importance of NADH-GOGAT to ammonium assimilation (Tamura et al. 2010) and nitrogen translocation (Tamura et al. 2011), and it also suggested that the decrease in grain numbers caused the yield reduction in the two *NADH-GOGAT* mutants (Tamura et al. 2010, 2011). The nitrogen which is stored in rice grains originates from photosynthetic organs (Mae and Ohira 1981). It is evident that the marked decrease in grain numbers in the mutants would have increased the amount of nitrogen available for each individual grain in the *NADH-GOGAT* mutants. This could explain the increased grain protein concentration in *nadh-gogat2*. The form in which nitrogen is transported to the mutant grains seems to be protein and amino acids. The free amino acids which accumulated in the mutant grains differed in composition between the mutants. Glutamate occupies a central position in amino acid metabolism in plants (Forde and Lea 2007). A loss of GOGAT activity could change the concentrations of several amino acids, including free glutamine (Table 1). Of the amino acids in the rice grain, glutamine accumulated to one of the highest concentrations, and it is also one of the substrates for the GOGAT reaction. GABA is synthesized from glutamate; therefore, we predicted a decrease in GABA concentration in the *NADH-GOGAT* mutants, but the concentration of GABA in the mutants was higher than that in 'Nipponbare'. It could be inferred, therefore, that the imbalance of the organic acid concentrations in the citrate cycle may influence the GABA concentration, since 2-oxoglutarate is another substrate for the GOGAT-catalyzed reaction. Unlike the forward genetic studies, we showed an increase in nitrogen compounds, including proteins and amino acids, in the *NADH-GOGAT* mutants.

Our results provide the first nutritional characterization of the grains of *NADH-GOGAT* mutants. An important question for future studies is how to improve the yield of *NADH-GOGAT* mutants while retaining the higher protein and amino acid concentrations. If achieved, this could eventually lead to the improvement of rice for human nutrition. In addition, the higher protein and amino acid concentrations of the mutant rice may have potential in the production of sweet sake and Shaoxing wine. *Tos17* is a retrotransposon found in the rice genome. Since it is an endogenous transposon, the Tos17 insertion lines are not categorized as transgenic plants, harboring an exogenous gene. The *Tos17* insertion lines used in this study could be used for hybridization with commercial rice cultivars to improve their grain protein, GABA and specific amino acid concentrations.

Methods

Plant material

Seeds of the *Tos17* insertion lines for *NADH-GOGAT1* (Tamura et al. 2010) and *NADH-GOGAT2* (Tamura et al. 2011) and their parent 'Nipponbare' were used in this study. Seeds of 'Nipponbare' and *NADH-GOGAT* mutants were sterilized in 2% sodium hypochlo-

rite solution for 20 min and germinated on moistened filter paper (No.2, 82 mm; Toyo Roshi Kaisha, Ltd., Tokyo, Japan) for 42–66 h at 30°C in Petri dishes (GD90-15; AS ONE Corporation, Osaka, Japan). The seeds were sown in cell trays (Cell-box, 25 holes, 285×285×56.5 mm, Meiwa Co., Ltd., Aichi, Japan) containing a synthetic cultural soil (N:0.7 g, P_2O_5:1.2 g, K_2O:0.6 g/1kg, Type-L, Sanken-Soil Corporation, Iwate, Japan), with one seed per cell, and grown in the greenhouse under natural light for 42 d. Seedlings were then transplanted in the paddy field in Amamiya. The density of the seedling in the paddy field was one to 0.003 m^2. The average monthly temperature at Sendai from June to September on 2014 was 20.6, 23.7, 24.6, and 20.5°C, the mean monthly rainfall was 242, 123, 133, and 112 mm, and the monthly hours of daylight was 137.9, 159.2, 137.9, and 192.8 h (Japan Meteorological Agency, Tokyo, Japan). Fully mature and full-sized grains of 'Nipponbare' and the two *NADH-GOGAT* mutants were selected by soaking in a NaCl solution (density = 1.06 g•ml^{-1}) with gentle shaking using a magnetic stirrer for 1 min; mature seeds sank under these conditions. Fifty seeds were randomly selected, dehusked and weighed. Only those grains whose weights were within the range mean ± 0.5 mg were used for further analysis.

Determination of NADH-GOGAT activity

The activity of NADH-GOGAT in rice grains was determined according to the work previously published (Hayakawa et al. 1990). Rice grains were used 24 h after imbibition of water. Protein was extracted from 100 rice grains with 3 mL extraction buffer (50 mM KH_2PO_4-KOH, pH7.5, 0.2% beta-mercaptoethanol, 1 mM EDTA, 2 mM 2-oxoglutarate, 2 mM phenylmethylsulphonyl fluoride, 20 μM leupeptin 500 μM 4-(2-aminoethyl)-benzensulfonyl fluoride, 100 mM KCl, 0.05% Triton X-100) by grinding with a motor and pestle. After the centrifugation at 15,000 × g at 4 °C for 20 min, the supernatant was desalted on a Sephadex G-25 PD-10 column (GE health care Japan K. K., Tokyo, Japan) in order to prepare crude extracts. The protein concentration was determined by the method described by Bradford (1976). The crude extracts were mixed in 50 mM KH_2PO_4-KOH, pH7.5, 10 mM glutamine, 10 mM 2-oxoglutarate, 0.16 mM NADH and incubated at 30 °C for 20 min, and then 100 °C for 30 sec in order to stop the reaction. Glutamate was trapped on an anion exchange column (AG1X8, Bio-Rad), and eluted with 0.5 M acetate after removing glutamine. The concentration of glutamate was determined by the ninhydrin procedure (Garrels et al. 1972). A unit of enzyme is defined as that amount that synthesizes 1 μmol glutamate per min at 30°C.

Protein extraction and SDS-PAGE

One grain of each of 'Nipponbare' and the two *NADH-GOGAT Tos17* insertion lines was ground separately into a fine powder in a 3-mL tube with a Multi-Beads Shocker (Yasui-Kikai Co. Ltd, Osaka, Japan) at 2,000 rpm, for 10 s per cycle, for four cycles. The grain had been selected to be mature and full-sized as described in section 2.1. For total protein extraction, 550 μL of extraction buffer (125 mM Tris-HCl, pH 6.8, 2% (w/v) sodium dodecyl sulfate (SDS), 10% (v/v) glycerol; Hayakawa et al. 1993) was added to the seed powder and homogenized again with the Multi-Beads Shocker at 2,000 rpm, for 10 s per cycle, for four cycles. The homogenate was centrifuged at 15,000 x g for 30 min at 25°C. The supernatant was used for further analysis. The protein concentration of the supernatant was determined using the RC DC Protein Assay Kit (Bio-Rad Laboratories Inc., Tokyo, Japan), with bovine serum albumin as a standard. Total seed protein was separated by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) on 14% (w/v) acrylamide running gels. After electrophoresis, the gels were stained with Coomassie Brilliant Blue R-250. The band

images were scanned and quantified using Image J (Schneider et al. 2012).

Determination of free amino acid concentration

Ten seeds from each line were placed in a 3-mL tube (Yasui-Kikai Co. Ltd, Osaka, Japan) with metal beads and powdered with a Multi-Beads Shocker at 2,000 rpm, for 10 s per cycle, and a total of four cycles. Powdered samples were suspended in five volumes of 10 mM HCl and homogenized in a Multi-Beads Shocker again at 2,000 rpm, for 10 s per cycle, and a total of four cycles, before being centrifuged at 15,000 x g for 30 min at room temperature. The supernatant was transferred to a prepared Vivaspin 500 Polyethersulfone 3,000 MWCO 1.5 mL tube (Sartorius Lab Instruments, Göttingen, Germany) for sample concentration, and centrifuged at 15,000 x g for 20 min at room temperature. The flow-through was used for labeling. The AccQ•Tag Ultra Derivatization Kit (Nihon Waters K. K., Tokyo, Japan) was used for derivatization of free amino acids and free ammonium (Konishi et al. 2014, 2017). The amino acids were derivatized with 6- aminoquinolyl-N-hydroxysuccinimidyl carbamate. AccQ•Tag-labeled samples were kept at room temperature and amino acid concentrations were determined using an ACQUITY Ultra-Performance Liquid Chromatography (UPLC) H-Class system (Nihon Waters K. K., Tokyo, Japan).

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Disclosures

Conflicts of interest: No conflicts of interest are declared.

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References

Akama, K., Kanetou, J., Shimosaki, S., Kawakami, K., Tsuchikura, S., Takaiwa, F., 2009. Seed-specific expression of truncated OsGAD2 produces GABA-enriched rice grains that influence a decrease in blood pressure in spontaneously hypertensive rats. *Transgenic Res* 18, 865-876.

Bradford, M.M., 1976. A rapid and sensitive method for quantitation of microgram quantities of protein utilizing principle of protein-dye binding. *Anal. Biochem.* 72, 248-254.

Forde, B.G., Lea, P.J., 2007. Glutamate in plants: metabolism, regulation, and signalling. *Journal of Experimental Botany* 58, 2339-2358.

Gadaleta, A., Nigro, D., Giancaspro, A., Blanco, A., 2011. The glutamine synthetase (GS2) genes in relation to grain protein content of durum wheat. *Functional & Integrative Genomics 11*, 665-670.

Garrels, J.I., Elgin, S.C.R., Bonner, J., 1972. A histone protease of rat liver chromatin. *Biochemical and Biophysical Research Communications* 46, 545-551.

Hayakawa, T., Kamachi, K., Oikawa, M., Ojima, K., Yamaya, T., 1990. Response of glutamine-synthetase and glutamate synthase isoforms to nitrogen-sources in rice cell-cultures. *Plant and Cell Physiology* 31, 1071-1077.

Hayakawa, T., Yamaya, T., Mae, T., Ojima, K., 1993. Changes in the content of 2 glutamate synthase proteins in spikelets of rice (*Oryza sativa*) plants during ripening. *Plant Physiology* 101, 1257-1262.

Kojima, S., Konishi, N., Beier, M.P., Ishiyama, K., Maru, I., Hayakawa, T., Yamaya, T., 2014. NADH-dependent glutamate synthase participated in ammonium assimilation in Arabidopsis root. *Plant Signaling & Behavior* 9,

e29402.

Komatsuzaki, N., Tsukahara, K., Toyoshima, H., Suzuki, T., Shimizu, N., Kimura, T., 2007. Effect of soaking and gaseous treatment on GABA content in germinated brown rice. *Journal of Food Engineering* 78, 556-560.

Konishi, N., Ishiyama, K., Matsuoka, K., Maru, I., Hayakawa, T., Yamaya, T., Kojima, S., 2014. NADH-dependent glutamate synthase plays a crucial role in assimilating ammonium in the Arabidopsis root. *Physiologia Plantarum* 152, 138-151.

Konishi, N., Ishiyama, K., Beier, M.P., Inoue, E., Kanno, K., Yamaya, T., Takahashi, H., Kojima, S., 2017. Contribution of two glutamine synthetase isozymes to ammonium assimilation in Arabidopsis roots. *Journal of Experimental Botany* 68, 613-625.

Konishi, N., Saito, M., Imagawa, F., Kanno, K., Yamaya, T., Kojima, S. 2018. Cytosolic glutamine synthetase isozymes play redundant roles in ammonium assimilation under low-ammonium conditions in roots of *Arabidopsis thaliana*. *Plant and Cell Physiology* 59, 601-613.

Mae, T., Ohira, K., 1981. The remobilization of nitrogen related to leaf growth and senescence in rice plants (*Oryza sativa* L). *Plant and Cell Physiology* 22, 1067-1074.

Nigro, D., Blanco, A., Anderson, O.D., Gadaleta, A., 2014. Characterization of ferredoxin-dependent glutamine-oxoglutarate amidotransferase (Fd-GOGAT) genes and their relationship with grain protein content QTL in wheat. *Plos One* 9.

Nigro, D., Fortunato, S., Giove, S., Mangini, G., Yacoubi, I., Simeone, R., Blanco, A., Gadaleta, A., 2017. Allelic variants of glutamine synthetase and glutamate synthase genes in a collection of durum wheat and association with grain protein content. *Diversity* 9, 52.

Peng, B., Kong, H., Li, Y., Wang, L., Zhong, M., Sun, L., Gao, G., Zhang, Q., Luo, L., Wang, G., Xie, W., Chen, J., Yao, W., Peng, Y., Lei, L., Lian, X., Xiao, J., Xu, C., Li, X., He, Y., 2014. OsAAP6 functions as an important regulator of grain protein content and nutritional quality in rice. *Nature Communications* 5, 4847.

Saikusa, T., Horino, T., Mori, Y., 1994. Accumulation of gamma-aminobutyric acid (GABA) in the rice germ during water soaking. *Bioscience, Biotechnology, and Biochemistry* 58, 2291-2292.

Schneider, C.A., Rasband, W.S., Eliceiri, K.W., 2012. NIH Image to ImageJ: 25 years of image analysis. *Nature Methods* 9, 671-675.

She, K.C., Kusano, H., Koizumi, K., Yamakawa, H., Hakata, M., Imamura, T., Fukuda, M., Naito, N., Tsurumaki, Y., Yaeshima, M., Tsuge, T., Matsumoto, K., Kudoh, M., Itoh, E., Kikuchi, S., Kishimoto, N., Yazaki, J., Ando, T., Yano, M., Aoyama, T., Sasaki, T., Satoh, H., Shimada, H., 2010. A novel factor FLOURY ENDOSPERM2 is involved in regulation of rice grain size and starch quality. *Plant Cell* 22, 3280-3294.

Suzuki, Y., Kawazu, T., Koyama, H., 2004. RNA isolation from siliques, dry seeds, and other tissues of *Arabidopsis thaliana*. *BioTechniques* 37, 542-544.

Suzuki, Y., Ohkubo, M., Hatakeyama, H., Ohashi, K., Yoshizawa, R., Kojima, S., Hayakawa, T., Yamaya, T., Mae, T., Makino, A., 2007. Increased rubisco content in transgenic rice transformed with the 'sense' rbcS gene. *Plant and Cell Physiology* 48, 626-637.

Tamura, W., Hidaka, Y., Tabuchi, M., Kojima, S., Hayakawa, T., Sato, T., Obara, M., Kojima, M., Sakakibara, H., Yamaya, T., 2010. Reverse genetics approach to characterize a function of NADH-glutamate synthase1 in rice

plants. *Amino Acids* 39, 1003-1012.

Tamura, W., Kojima, S., Toyokawa, A., Watanabe, H., Tabuchi-Kobayashi, M., Hayakawa, T., Yamaya, T., 2011. Disruption of a novel NADH-glutamate synthase2 gene caused marked reduction in spikelet number of rice. *Frontiers in Plant Science* 2, 1-9.

Wang, G., Sun, X.L., Wang, G.F., Wang, F., Gao, Q., Sun, X., Tang, Y.P., Chang, C., Lai, J.S., Zhu, L.H., Xu, Z.K., Song, R.T., 2011. Opaque7 encodes an acyl-activating enzyme-like protein that affects storage protein synthesis in maize endosperm. *Genetics* 189, 1281-1295.

Ye, G.Y., Liang, S.S., Wan, J.M., 2010. QTL mapping of protein content in rice using single chromosome segment substitution lines. *Theoretical and Applied Genetics* 121, 741-750.