Effect of late nitrogen fertilizer application on free amino acid content in brown rice and germinated brown rice

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Abstract

Effects of topdressing nitrogen (N) at the panicle formation stage on the content of free amino acids, especially gamma-aminobutyric acid (GABA), of germinated brown rice (GBR) was examined in rice variety Koshihikari grown in pots and in the field. In potted plant experiments (2004), rice seedlings (*Oryza Sativa* L.) were cultivated in Wagner pots (1/5000 a), and N topdressing was applied in concentrations of 2 g m^{-2} N or 4 g m^{-2} N, 15 days before the heading date (conventional treatment), 5 days before the heading date, and on the heading date. The protein content in brown rice (BR) and the GABA content in GBR in plants topdressed with N at 5 days before the heading date and on the heading date were significantly higher than those of the conventional treatment, and a positive correlation was observed between protein content in BR and GABA content in GBR ($r=0.549$, $p<0.01$). In field experiments (2005), N was topdressed at concentrations of 3 g m^{-2} N or 6 g m^{-2} N, 10 days before heading date (control), on the heading date, and 5 days after the heading date. The protein content of BR increased as the time of application was delayed and the amount of nitrogen applied increased. The GABA content in GBR increased with increasing amounts of applied N, and was the highest when applied on the heading date (1.79～1.84 μmol g^{-1}). In contrast, both potted and field grown plants showed a reduction in culm length, panicle length and yield when N was applied on or after the heading date, compared with that of the control. These results suggested that abundant N application on the heading date is useful for increasing the content of GABA in GBR.

Key words: nitrogen, topdressing, amino acid, gamma-aminobutyric acid (GABA), germinated brown rice.

INTRODUCTION

Gamma-aminobutyric acid (GABA), a four-carbon non-protein bound amino acid, is found in all prokaryotic and eukaryotic organisms as a significant component of the free amino acid pool (Bown and Shelp 1997). Generally, the content of GABA in plant tissues is maintained at low levels (Rhodes *et al.* 1986; Fougére *et al.* 1991), and increases rapidly several-fold in response to environmental stress such as mechanical stimulation, mechanical damage, cold shock (Wallace *et al.* 1984), heat shock (Mayer et al. 1990), hypoxia (Tsushida and Murai 1987), cytosolic acidification (Crawford *et al.* 1994), water stress (Bolarin *et al.* 1995), and phytohormones (Ford et al. 1996). In soybean leaves (*Glycine max*), Wallace *et al.* (1984) reported that cold or mechanical stimulation increased GABA levels 20 to 40-fold within 5 min. Under anaerobic conditions, GABA content in tea leaves (*Camellia sinensis* cv Yabukita) accumulated approximately 8.9-fold (Tsushida and Murai 1987). It was also found that water soaking of rice flours brought about remarkable increases in GABA content (Saikusa *et al.* 1994a), and accumulation of GABA in rice seedlings was induced by anoxia (Aurisano *et al.* 1995). Because GABA accumulation in response to a variety of stimuli has been observed in various plants, it is presumed that GABA plays a universal role in higher plants, although, until now, little scientific evidence regarding its functionality has been reported.

GABA is well known to have beneficial effects for human health such as the reduction of blood pressure (Elliott and Honniger 1959; Omori *et al.* 1987) and the improvement of a menopausal disorder and depression (Okada *et al.* 2000). Gabaron Tea, which is produced by the incubation of fresh tea leaves in anaerobic condition, was the first GABA enhanced food marketed in Japan, and since its introduction other GABA enriched foods have come into practical use. For example, in 1999, GABA enriched GBR was produced by soaking BR in water until the grains elongated to 0.5 - 1.0 mm, which resulted in production of a new cereal for Japanese markets. However, the GABA content in GBR varies significantly in grains cultivated in different regions of the country and in different years. Therefore, it is necessary to determine cultivation methods that effectively and consistently enhance GABA content in GBR. Several experiments have suggested that rice varieties with giant embryos (Maeda *et al.* 2001 and those adapted to cold climates (Akama *et al.* 2001) are useful for production of GABA enriched GBR. However, practice methods using manure to enrich GABA content in GBR have not been elucidated.

In higher plants, it was shown that GABA was synthesized and metabolized through the GABA shunt. In this pathway, GABA is primarily synthesized directly through

irreversible α-decarboxylation of L-glutamate (L-Glu) by glutamate decarboxylase (GAD). GABA transaminase (GABA-T) then catalyses the reversible conversion of GABA to succinic semialdehyde using either pyruvate or α-ketoglutarate as amino an acceptor. The last step in the GABA shunt is catalyzed by succinic semialdehyde dehydrogenase (SSADH), which irreversibly oxidizes succinic semialdehyde to succinate (Snedden *et al.* 1995; Bown and Shelp 1997). It was reported that in roots of legumes, GABA synthesized successfully under such conditions was a product of reduced synthesis or enhanced degradation of protein (Satya narayan and Nair 1990). Saikusa. *et al.* (1994b); further suggesting that Glu was produced through proteolysis and subsequently served as a substrate for GABA synthesis during water soaking of rice. Therefore, BR containing high levels of protein yielded high levels of GABA via proteolytic degradation and concomitant increases in Glu for use as a synthetic substrate. However, the relationship between BR protein content and GBR GABA content has not been elucidated.

The protein content of BR was strongly affected by nitrogen application. Late application of N induced higher protein content in BR than did conventional nitrogen applications (Patrick and Hoskins 1974; Perez *et al.* 1996). Spraying urea on rice leaves at the flowering stage also increased the content of protein in rice grain (Nishizawa *et al.* 1977). Although the importance of rice protein as a predominate dietary source for many people worldwide is well (Juliano *et al.* 1990), it was also shown that when protein content in rice increased, the eating quality was reduced (Ishima *et al.* 1974). Thus, guidelines for rice cultivation in Japan did not recommend late N application. However, in view of GBR GABA content, late topdressing of N to increase the GABA content in GBR may be a more useful method for cultivation than conventional N application protocols.

In the present study, we examined the effect of topdressing at the panicle formation stage on the content of free amino acids, especially GABA, in GBR, and elucidated the optimum time and amount of late N application for production of GABA enriched BR.

MATERIALS AND METHODS

Plant materials

A rice variety Koshihikari used in potted plant and field plant experiments was obtained from the Center for Education and Research of Field Science of Shizuoka University (Fujieda, Japan).

Pot experiment

Potted plant experiments were conducted at the Faculty of Agriculture, Shizuoka University (Shizuoka, Japan) in 2004. Wagner pots (1/5000 a) were filled with paddy soil collected from the experimental field of Shizuoka University (Fujieda, Japan). Chemical fertilizer (N 4 g, P_2O_5 10 g and K_2O 15 g m^{-2}) was applied as a basal dressing on May 25, 2004. Forty-day old seedlings were transplanted into pots by hand on June 11. Pots were topdressed with ammonium sulfate 15 days before the heading date, 5 days before the heading date, or on the heading date. N was applied at 2 g m^{-2} N (conventional) or 4 g m^{-2} N, as determined in a previous report (Takebe, 1999). An abbreviated nomenclature was used to denote various nitrogen applications: N4200 (control), N4400, N4020, N4040, N4002 and N4004; N represents nitrogen treatment; successive numerals in each term denote the amount of nitrogen (g m^{-2}) applied as a basal dressing, at 15 days before the heading date, at 5 days before the heading date, and on the heading date, respectively. Rice plants were grown in a greenhouse until maturity, and harvested on September 17. Each treatment condition was performed in nine replicates. Panicle number, culm length and panicle length were measured at the time of harvest. Yield was evaluated after air drying. Soil from each cultivation pot was air-dried, milled, passed thorough a sieve (2 mm) and stored in an airtight bag at room temperature until analysis. After air drying, shoot and BR were lyophilized using a FDU-2000 lyophilizer (Tokyo Rikakikai Co., Tokyo, Japan), ground with an electrical mill, and stored at -20 ℃ until analysis. The total N content (T-N) of soil, shoot, and BR, and the content of GABA in BR and GBR were measured. All analyses were performed in triplicate.

Field experiment

Field experiments were carried out in a conventional paddy field at the Center for Education and Research of Field Science of Shizuoka University. The field had received yearly applications of N 72 kg, P_2O_5 144 kg and K_2O 68 kg ha^{-1} $year^{-1}$. As a basal dressing, N 4 g m^{-2}, P_2O_5 4.8 g m^{-2} and K_2O 4 g m^{-2} were applied on May 28. Thirty eight-day old seedlings were transplanted individually, by hand to a paddy field on June 3. Since late nitrogen treatment in potted plant experiments was shown to increase the GABA content in GBR, plants grown in field experiments were treated at a time point 5 days after the heading date. Topdressing was carried out 10 days before the heading date, on the heading date, or 5 days after the heading date. The amount of N applied was 3 g m^{-2} (conventional) or 6 g m^{-2} (two times conventional) according to the conventional method of this region (1.5-fold higher than that of pot experiment) as ammonium sulfate. The treatment

nomenclature used followed the same patter as that for the potted plants experiment: N4300 (control), N4600, N4030, N4060, N4003 and N4006. Plants were cultivated according to a conventional method of proper area. Plot dimensions for each treatment were 1.62 m^2, and experiments were performed in three replicate. About thirty-five days after the heading date, 14 plants located in the center of each plot were measured for tiller number, culm length and panicle length. Culm lengths and panicle lengths were determined using the longest culm of each hill. After harvest, the grain yield was evaluated after approximately 2 weeks of air drying. The paddy soil of each treatment condition was sampled (0-15 cm and 15-30 cm in depth), air-dried, milled, passed through a sieve (2 mm), and stored in an airtight bag at room temperature until analysis. The shoot and BR were lyophilized, ground with an electrical mill, and stored at -20 ℃ until analysis. T-N content of paddy soil, shoot, and BR, and free amino acids of BR and GBR were measured. All analyses were performed in triplicate.

Preparation of germinated brown rice (GBR)

For preparation of GBR, an aliquot of stored BR was germinated in a microcomputer electric germination appliance HP-30 HatsugaBijin (Takekoshi Co., Niigata, Japan) at 32 ℃ for 21 hours (bud elognated from 0.5 to 1.0 mm). Then, GBR was immediately freeze-dried and ground with an electrical mill. The resulting GBR flour was stored at -20 ℃ until analysis.

Measurement of T-N and free amino acids

The T-N content of soil, shoot and BR from plants in the pot and the field experiments were determined with a NC analyzer (SUMIGRAPH NC-95, Sumika Chemical Analysis Service, Tokyo, Japan). The protein content was calculated from the T-N content value using an exchange rate of 5.95.

Free amino acids of BR and GBR were extracted according to the modified method of Abe *et al.* (2000). An amount of 100 mg BR or GBR flour was extracted with 1 mL extraction buffer (acetonitrile and 0.1% trifluoroacetic acid (1:1 v/v)) by rotary shaker (130 rpm) at room temperature for 1 hour. The mixture were centrifuged at 10,000 ×g at 4 ℃ for 10 min, and the supernatant was collected. The residue was extracted again in the same manner. The combined supernatant from both extraction was filtered through a 0.45 ?m membrane filter (Advantec Co., Ltd., Tokyo, Japan), and samples were stored at -20 ℃ until HPLC analysis.

The content of glutamate (Glu), glutamine (Gln), aspartic acid (Asp), asparagine (Asn),

serine (Ser), alanine (Ala) and GABA in sample solution was determined according to the method of Goto *et al.* (1993) using glycylglycine as an internal standard. All analyses were performed in triplicate. In the potted plants experiment, only the content of GABA in GBR was analyzed.

The reagents for amino acids analysis standard were obtained from Wako Pure Chemical Industries Co., Ltd. (Osaka, Japan). HPLC grade acetonitrile was purchased from Kanto Chemical (Tokyo, Japan). All other reagents used in this study were of analytical grade and were used without further purification.

Statistical analysis

Differences between treatments were determined using an analysis of variance. The means were compared according to the Tukey's multiple range test ($P < 0.05$).

RESULTS AND DISCUSSION

Panicle number, culm length, panicle length and yield of BR measured in the potted plants experiment are shown in Table 1. Panicle number was not affected by the amount of N applied or the time of N application. However, culm length was reduced in plants that received N application on the heading date (N4002 and N4004) compared to those that received N application before the heading date. Panicle length and yield of BR tended to decline with later applications of N. In the field plants experiment, panicle number did not differ significantly between treatments (Table 2).

However, there was a significant difference in culm length between plants that received N application after the heading date and those that were treated 10 days before the heading date. Also, panicle length was greatest in plants receiving conventional (N4300) N application, but was lowest in plants receiving N application 5 days after the heading date (N4003). Thus, N application after the heading date reduced panicle length, as well as culm length. Yield was highest in plants grown in conventional plot (N4300) conditions, but was lowest in plants from plots where 6 g m^{-2} N was applied on the heading date (N4060). Thus, the yield was reduced by application of N after the heading date. Takebe (1999) indicated that culm length in plants from plots in which N was applied 25 days before the heading date was higher than that those from plots in which N was applied on the heading date. It appears that delaying the application of N reduced the yield via the concomitant reduction in panicle and culm length.

Table 1 Comparison of panicle number, culm length, panicle length and yield in brown rice grown in pots

Treatment[1)]	Panicle number (plant^{-1})	Culm length (cm)	Panicle length (cm)	Yield (g m^{-2})
N 4200	6.3±1.0 [a]	74.7±3.6 [ab]	18.8±1.6 [a]	479±80 [a]
N 4400	5.4±0.7 [a]	78.3±4.2 [a]	18.6±1.1 [a]	397±82 [a]
N 4020	5.7±0.7 [a]	78.0±4.6 [a]	18.0±1.3 [a]	401±75 [a]
N 4040	5.7±1.2 [a]	77.3±5.5 [a]	17.6±1.0 [a]	402±74 [a]
N 4002	6.2±0.8 [a]	72.4±4.6 [ab]	18.2±1.9 [a]	419±47 [a]
N 4004	5.7±0.9 [a]	69.6±4.8 [b]	17.6±1.0 [a]	343±99 [a]

Koshihikari was cultivated in Wagner pots and treated with various applications of nitrogen (2004).
1) Nitrogen treatment nomenclature: N4200, N4400, N4020, N4040, N4002 and N4004; N represents nitrogen treatment; successive numerals in each term denote the amount of nitrogen (g m^{-2}) applied as a basal dressing, at 15 days before heading date, at 5 days before heading date and on the heading date, respectively. Data are means ± SD, n=9. Date followed by a letter different than that of other letters within the same column are significantly different at the 5% level (Tukey).

Table 2 Comparison of panicle number, culm length, panicle length and yield in brown rice grown in fields

Treatment[1)]	Panicle number (plant^{-1})	Culm length (cm)	Panicle length (cm)	Yield (g m^{-2})
N 4300	13.3 ± 1.5 [a]	88.9 ± 0.6 [a]	21.2 ± 0.0 [a]	564 ± 30 [a]
N 4600	14.4 ± 0.2 [a]	89.5 ± 1.2 [a]	21.0 ± 0.0 [ab]	543 ± 6 [ab]
N 4030	13.5 ± 0.9 [a]	84.4 ± 1.4 [b]	20.3 ± 0.4 [b]	505 ± 12 [ab]
N 4060	12.6 ± 0.7 [a]	85.4 ± 0.2 [b]	20.3 ± 0.5 [b]	492 ± 31 [b]
N 4003	14.7 ± 0.7 [a]	86.0 ± 0.6 [b]	20.0 ± 0.4 [b]	510 ± 33 [ab]
N 4006	14.2 ± 0.8 [a]	83.5 ± 1.8 [b]	20.3 ± 0.5 [b]	522 ± 19 [ab]

Koshihikari was cultivated in a paddy field and treated with various applications of nitrogen (2005).
1) Nitrogen treatment nomenclature: N4300, N4600, N4030, N4060, N4003 and N4006; N represents nitrogen treatment; successive numerals in each term denote the amount of nitrogen (g m^{-2}) applied as a basal dressing, at 10 days before heading date, on the heading date, and 5 days after the heading date, respectively. Data are means ± SD, n=3. Date followed by a letter different than that of other letters within the same column are significantly different at the 5% level (Tukey).

T-N content for soil and shoots (culm and leaves) of potted plants is shown Table 3. Maximum T-N content was observed in soils treated with 4 g m^{-2} N, 5 days before the heading date (N4040; 2.4 mg g^{-1} DW). However, T-N content in shoots from potted plants increased as the amount of N applied increased. The protein content of BR was lowest in plants receiving conventional N treatment (N4200; 51.2 mg g^{-1} DW) (Table 4).

Table 3 Total nitrogen content of soil and shoots in plants grown in pots.

Treatment[1)]	Soil in pot (mg g^{-1}DW)	Shoot (leaf and culm) (mg g^{-1}DW)
N 4200	2.2 ± 0.1 [b]	5.2 ± 1.1 [ab]
N 4400	2.2 ± 0.0 [ab]	5.3 ± 0.7 [ab]
N 4020	2.3 ± 0.0 [ab]	5.0 ± 0.8 [b]
N 4040	2.4 ± 0.1 [a]	5.3 ± 0.8 [ab]
N 4002	2.3 ± 0.1 [ab]	5.0 ± 0.8 [b]
N 4004	2.3 ± 0.1 [ab]	6.7 ± 1.5 [a]

Koshihikari was cultivated in Wagner pot and treated with various application of nitrogen (2004).

1) Nitrogen treatment nomenclature: N4200, N4400, N4020, N4040, N4002 and N4004; N represents nitrogen treatment; successive numerals in each term denote the amount of nitrogen (g m^{-2}) applied as a basal dressing, at 15 days before heading date, at 5 days before heading date and on the heading date, respectively. Data are the mean ± SD, n=9. Date followed by a letter different than that of other letters within the same column are significantly different at the 5% level (Tukey).

Table 4 BR protein content and GBR GABA content in plants grown in pots

Treatment[1)]	Protein content of BR (mg g^{-1}DW)	GABA content of GBR (μmol g^{-1}DW)
N 4200	51.2 ± 2.4 [b]	2.00 ± 0.14 [a]
N 4400	53.0 ± 2.4 [b]	2.25 ± 0.08 [a]
N 4020	62.5 ± 3.6 [a]	2.62 ± 0.41 [a]
N 4040	62.5 ± 4.2 [a]	2.35 ± 0.35 [a]
N 4002	59.5 ± 3.0 [a]	2.79 ± 0.69 [a]
N 4004	61.3 ± 6.5 [a]	2.99 ± 0.61 [a]

Koshihikari was cultivated in Wagner pots (2004).

1) Nitrogen treatment nomenclature: N4200, N4400, N4020, N4040, N4002 and N4004; N represents nitrogen treatment; successive numerals in each term denote the amount of nitrogen (g m^{-2}) applied as a basal dressing, at 15 days before heading date, at 5 days before heading date and on the heading date, respectively. Data are means ± SD, n=3. Date followed by a letter different than that of other letters within the same column are significantly different at the 5% level (Tukey). BR, brown rice; GBR, germinated brown rice.

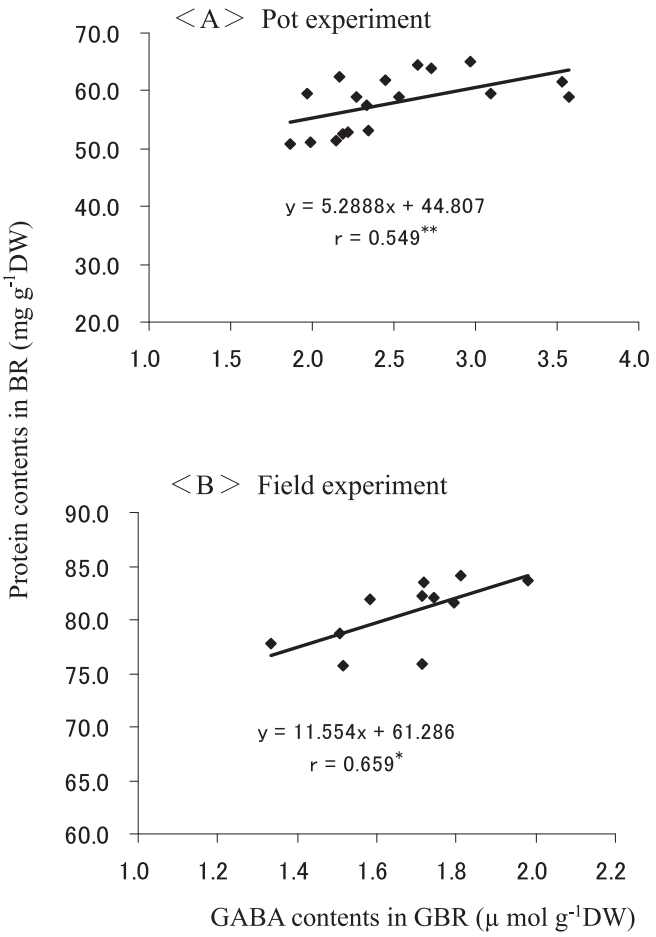

Figure 1. Relationship between protein content in BR and GABA content in GBR of plants grown in pots and field grown plants.

*P<0.05, **P<0.01. BR, brown rice; GBR, germinated brown rice.
＜A> Pot experiment (n=21). <B> Field experiment (n=11), Date were shown at 10 days before heading date and heading date.

Patric and Hoskins 1974, Nishizawa *et al.* 1977, Takebe 1999). Although statistical significance was not observed, the content of GABA in GBR prepared from plants that received N treatment on the heading date. (N4004; 2.99 μmol g-1 DW) was 1.5-fold higher than that of plants receiving conventional N treatment (N4200; 2.00 μmol g^{-1} DW). A positive correlation between BR protein content and GBR GABA content was observed (r=0.549, p<0.01) (Fig. 1A). These data suggested the possibility that late N application increased the content of GABA in GBR.

In soil from the field plants experiment, the difference in T-N content between the upper (0～15 cm) and lower (15～30 cm) soil layers of various treatments was negligible, except in the lower (15～30 cm) soil layer of N4030 and N4006 plots (Table 5). The T-N content in shoots increased with the delay of N treatment and the amount of N applied, showing the highest and lowest values in plants that received 6 g m^{-2} N, 5 days after the heading

date (N4006; 12.2 mg g^{-1} DW) and the conventional treatment (4300; 9.0 mg g^{-1} DW), respectively. Therefore, in field grown plants as well as in potted plants, the BR protein content increased with the delay of N application, and the amount of N applied.

The BR and GBR free amino acid content from plants grown in the field experiment are shown in Table 6. No significant difference was observed in free amino acid content among BR grown under various treatment conditions, despite a 1.5 fold increase in the amount of N applied compared to the amount used in the potted plants experiment. These results indicated that the content of free amino acid in BR was not affected by the timing of application nor the amount of N applied, as shown in a previous report (Takebe 1999). In GBR, the GABA content was highest when the plants were topdressed on the heading date (1.79〜1.84 μmol g^{-1} DW). The GABA content in GBR from plants receiving N application 5 days after the heading date was lower than that for plants treated on the heading date; however, the protein content was highest in BR from these plants. A positive correlation was observed between the content of protein in BR and GABA in GBR when N was applied before or on the heading date (Fig. 1B). However, this positive correlation was obviated when measurements from plants receiving N treatment 5 days after the heading date were added to the above data for analysis. Thus, topdressing before or on the heading date increased the protein and GABA content in BR and GBR, respectively. However, topdressing after the heading date effectively increased the protein content in BR, but not the GABA content in GBR. Glu and Ala content in GBR from plants that received N application on the heading date were greater than that found in plants that received N treatment 5 days after the heading date. These results suggested that topdressing N after the heading date did not contribute to the increase in GBR Glu content via suppression of protein decomposition during germination. Thus, we purposed to analyze the change in protein and free amino acid content, and the activity of protease during germination, to evaluate the effect of N applied after the heading date on proteolysis.

Table 5. Total nitrogen and protein contents in soil, shoot and BR from field grown plants

Treatment[1)]	Tatal nitrogen contents			Protein contents
	Soil (paddy field)		Shoot (leaf and culm)	Brown rice
	0-15cm	15-30cm (mg g^{-1}DW)	(mg g^{-1}DW)	(mg g^{-1}DW)
N 4300	2.5 ± 0.1 [a]	2.1 ± 0.1 [a]	9.0 ± 0.3 [b]	79.7 ± 0.4 [b]
N 4600	2.6 ± 0.1 [a]	2.0 ± 0.1 [a]	9.0 ± 0.7 [b]	84.5 ± 0.1 [a]
N 4030	2.5 ± 0.1 [a]	1.8 ± 0.0 [b]	9.6 ± 1.6 [b]	77.9 ± 3.2 [b]
N 4060	2.5 ± 0.0 [a]	2.0 ± 0.0 [a]	11.3 ± 2.3 [ab]	84.5 ± 0.5 [a]
N 4003	2.6 ± 0.1 [a]	2.2 ± 0.1 [a]	11.4 ± 0.6 [a]	82.7 ± 1.3 [b]
N 4006	2.6 ± 0.2 [a]	1.8 ± 0.4 [b]	12.2 ± 1.6 [a]	87.5 ± 1.3 [a]

Koshihikari was cultivated in paddy field treated nitrogen applications in 2005.

1) Nitrogen treatment nomenclature: N4300, N4600, N4030, N4060, N4003 and N4006; N represents nitrogen treatment; successive numerals in each term denote the amount of nitrogen (g m^{-2}) applied as a basal dressing, at 10 days before heading date, on the heading date, and 5 days after the heading date, respectively. Data are means ± SD, n=3. Date followed by a letter different than that of other letters within the same column are significantly different at the 5% level (Tukey).

Table 6. Free amino acid content in BR and GBR from field grown plants treated with various application of nitrogen

Treatment[1)]	Free amino acid (μmol g^{-1})					
	Glu	Asn	Ser	Gln	Ala	GABA
N 4300	1.37 ± 0.14 [a]	0.67 ± 0.15 [a]	0.30 ± 0.03 [a]	0.07 ± 0.01 [a]	0.81 ± 0.08 [a]	0.31 ± 0.02 [a]
N 4600	1.57 ± 0.08 [a]	0.82 ± 0.17 [a]	0.35 ± 0.02 [a]	0.07 ± 0.02 [a]	0.95 ± 0.11 [a]	0.39 ± 0.07 [a]
N 4030	1.45 ± 0.24 [a]	0.81 ± 0.22 [a]	0.30 ± 0.05 [a]	0.06 ± 0.03 [a]	0.90 ± 0.13 [a]	0.42 ± 0.16 [a]
N 4060	1.55 ± 0.13 [a]	0.81 ± 0.18 [a]	0.30 ± 0.04 [a]	0.05 ± 0.01 [a]	0.92 ± 0.01 [a]	0.42 ± 0.11 [a]
N 4003	1.60 ± 0.14 [a]	0.94 ± 0.18 [a]	0.32 ± 0.03 [a]	0.06 ± 0.02 [a]	0.93 ± 0.07 [a]	0.39 ± 0.03 [a]
N 4006	1.35 ± 0.12 [a]	0.77 ± 0.03 [a]	0.29 ± 0.03 [a]	0.05 ± 0.01 [a]	0.88 ± 0.13 [a]	0.39 ± 0.06 [a]

＜B＞ GBR

Treatment[1)]	Free amino acid (μmol g^{-1})					
	Glu	Asn	Ser	Gln	Ala	GABA
N 4300	0.36 ± 0.03 [b]	0.17 ± 0.04 [b]	0.16 ± 0.02 [a]	0.13 ± 0.01 [b]	2.29 ± 0.09 [d]	1.45 ± 0.11 [b]
N 4600	0.42 ± 0.04 [ab]	0.27 ± 0.04 [a]	0.18 ± 0.02 [a]	0.17 ± 0.01 [a]	2.69 ± 0.17 [c]	1.67 ± 0.09 [a]
N 4030	0.47 ± 0.11 [a]	0.21 ± 0.05 [b]	0.18 ± 0.02 [a]	0.15 ± 0.02 [ab]	2.77 ± 0.14 [b]	1.79 ± 0.08 [a]
N 4060	0.41 ± 0.05 [ab]	0.21 ± 0.06 [b]	0.17 ± 0.02 [a]	0.16 ± 0.02 [ab]	2.90 ± 0.14 [a]	1.84 ± 0.13 [a]
N 4003	0.38 ± 0.02 [ab]	0.22 ± 0.03 [b]	0.16 ± 0.01 [a]	0.14 ± 0.01 [b]	2.68 ± 0.14 [c]	1.65 ± 0.05 [a]
N 4006	0.39 ± 0.01 [ab]	0.22 ± 0.04 [b]	0.16 ± 0.02 [a]	0.16 ± 0.01 [ab]	2.71 ± 0.10 [bc]	1.69 ± 0.14 [a]

Koshihikari was cultivated in a paddy field (2005).

1) Nitrogen treatment nomenclature: N4300, N4600, N4030, N4060, N4003 and N4006; N represents nitrogen treatment; successive numerals in each term denote the amount of nitrogen (g m^{-2}) applied as a basal dressing, at 10 days before heading date, on the heading date, and 5 days after the heading date, respectively. Date are mean ± SD, n=3. Date followed by a letter different than that of other letters within the same column are significantly different at the 5% level (Tukey). BR, brown rice; GBR, germinated brown rice; Glu, glutamate; Asn, asparagine; Ser, serine; Gln, glutamine; Ala, alanine; GABA, γ-aminobutyric acid.

GABA content in GBR increased with the amount of N applied, for all topdressing treatment dates. Figure 2 shows the relationship between yield for BR and GABA content in BR and GBR. As mentioned above, when N was applied on the heading date (N4030 and N4060), the content of GABA in GBR was highest, while the yield of BR was the lowest. This suggested that grain yield may decrease as a result of an increase in the amount of N applied.

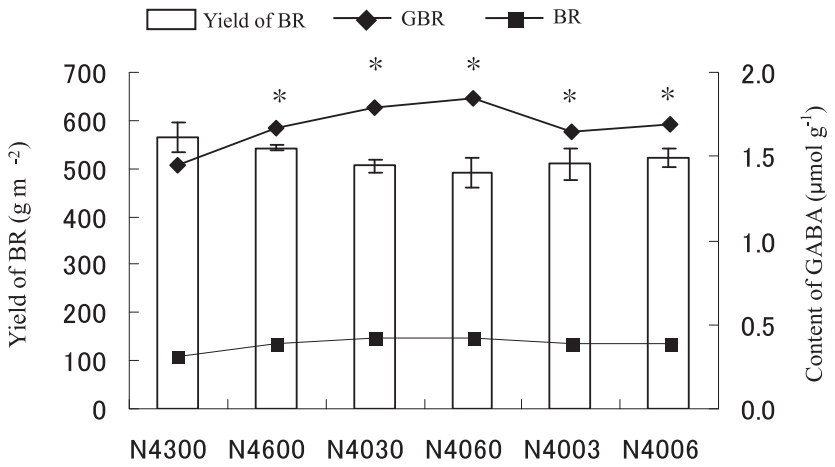

Figure 2. Yield of BR and GABA content in BR and GBR of field grown plants.

BR, brown rice; GBR, germinated brown rice.
Nitrogen treatment nomenclature: N4300, N4600, N4030, N4060, N4003 and N4006; N represents nitrogen treatment; successive numerals in each term denote the amount of nitrogen (g m-2) applied as a basal dressing, at 10 days before heading date, on the heading date, and 5 days after the heading date, respectively. Data are means ± S.D, n=3. * difference in GABA content between control (4300) and each treatment. Statistical significant at 5% level (Tukey) in GBR.

These results suggested that, in addition to delaying the date of topdressing until the heading date, applying amounts of N greater than that used for conventional culture is effective to increase the content of free amino acids, especially GABA, in GBR.
It was reported that consumption of fermented milk, which contains more than 10 mg 100 mL^{-1} GABA, induced the reduction of blood pressure when 100 mL was consumed daily for a period of 12 weeks (Kajimoto *et al.* 2003). Therefore, it was inferred that the intake of 10 mg GABA per day was beneficial to effect human health. In field grown plants, the content of GABA in GBR harvested from plots that received 6 g m^{-2} N on the heading date (N4060) increased to 1.84 μml g^{-1} DW, which was equal to 22.3 mg per 100 g GBR, containing 15% water. This value (1.84 μml g^{-1} DW) showed that 50 g of GBR harvested from the N4060 treatment group was adequate to supply an intake amount of 10 mg GABA. Thus, in this study, the content of GABA in GBR was enhanced using a simple

method, by manipulating the time of topdressing at panicle formation stage.

In current food production, several varieties of GBR rice, in addition to Koshihikari, are used. It is important to consider that topdressing conditions reported here, i.e., the time of N application and amount of N applied, were generated from experiments conducted using Koshihikari. However, these optimized parameters for the GABA content of GBR may vary for different rice varieties and according to the geographic regions of cultivation. Therefore, further investigation using various rice varieties and cultivation sources is needed to determine the most efficient and productive N application protocols for producing GABA enriched GBR.

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REFERENCES

Abe T, Matsuo T, Sasahara T 2000: Varietal difference of free amino acids and GABA contents in rice endsperm. *Breed. Res.*2 (Suppl. 2). 158 (in Japanese).

Akama K, Akihiro T, Kitagawa M, Takaiwa F 2001: Rice (*Oryza Sativa*) contains a novel isoform of glutamate decarboxylase that lacks an authentic calmpdulin-binding domain at the C-terminus. *Bioch. Biophy. Acta.* 1522, 143-150.

Aurisano N, Bertani A, Reggiani R 1995: Anaerobic accumulation of 4-aminobutyrate in rice seedlings; causes and significance. *Phytochem.*, 38, 1147-1150.

Bolarin MC, Santa-Cruz A, Cayuela E, Perez-Alfocea F 1995: Short-term solute changes in leaves and roots of cultivated and wild tomato seedlings under salinity. *J. Plant Physiol.* 147, 463-468.

Bown AW, Shelp BJ 1997: The Metabolism and Functions of γ-Aminobutyric Acid. *Plant Physiol.* 115, 1-5.

Crawford LA, Bown AW, Breitkreuz KE, Frédérique CG 1994: The synthesis of γ-Aminobutyric Acid in response to treatments reducing cytosolic pH. *Plant Physiol.* 104, 865-871.

Elliott KAC, Hobbiger F 1959: Gamma aminobutyric acid: Circulatory and respiratory effects in different spiecies; re-investigetion of the anti-strychnine- action in mice. *J. Physiol.*, 146, 70-84.

Ford Y-Y, Ratcliffe RG, Robins RJ 1996: Phytohormone-induced GABA production in transformed root cultures o *Datura stramonium*: an *in vivo* ^{15}N NMR study. *J. Exp Bot.*, 47, 811-818.

Fougère F, Rudulier DL, Streeter JG 1991: Effects of salt stress on amino acid, organic acid and carbohydrate composition of roots, bacteroids, and cytosol of alfalfa (*Medicago* sativa L.). *Plant Physiol.* 96, 1228-1236.

Goto T, Horie H, Mukai T 1993: Analysis of major amino acids in green tea by high-performance liquid chromatography coupled with OPA precplimn derivatization. *Tea research J.*, 77, 29-33 (in Japanese).

Ishima T, Taira H, Taira H, Mikoshiba K 1974: Effect of nitrogenous fertilizer application and protein content in milled rice on olganoleptic quality of cooked rice. *Rep. Nat'l. Food Res. Inst.*, 29, 9-15 (in Japanese with English summary).

Juriano BO, Perez CM, Kaosa-Ard M 1990: Grain quality characteristics of export rices in selected markets. *Cereal Chem.*, 67, 192-197.

Kajimoto O, Hirata H, Nishimura A 2003: Hypotensive action of novel fermented milk containing γ-ami nobutyric acid (GABA) in subjects with mild or moderate hypertension. *J.Nutritional Food*, 6, 51-64. (in Japanese with English summary).

Maeda H, Nemoto H, Iida S, Ishii T, Nakagawa N, Hoshino T, Sakai M, Okamoto M, Shinoda H, Yoshida T 2001: A new variety with giant embryos, “Haiminori”. *Breed.Scie.* 51, 211-213.

Mayer RR, Cherry JH, Rhodes D 1990: Effects of heat shock on amino acid metabolism of cowpea cells. *Plant Physiol.* 94, 796-810.

Nishizawa N, Kitahara I, Noguchi T, Hareyama S, Honjo K 1977: Protein quality of high protein rice obtained by spraying urea on leaves before harvest. *Agric. Biol.Chem.*, 41, 477-485.

Okada T, Sugisita A, Murakami H, Saikusa T, Horino T, Onoda A, Kajimoto O, Takahashi R, Takahashi T 2000: Effect of the defatted rice germ enriched with GABA fir sleeplesseness, depression, autonomic disorder by oral administration. *Nippon Shokuhin Kagaku Kogaku Kaishi.* 47, 596-603 (in Japanese with English summary).

Omori M, Toshiko Y, Okamoto J, Tsushida T, Murai T, Higuti M 1987: Effect of anaerobically treated tea (Gabaron Tea) on blood pressure of spontaneously hypertensive rats. *Nippon Nogeikagaku Kaishi.* 61, 1449-1451 (in Japanese with English summary).

Patrick RM, Hoskins FH 1974: Protein and amino acid content of rice as affected by application of nitrogen fertilizer. *Cereal Chem.*, 51, 84-95.

Perez CM, Juriano BO, Liboon SP, Alcantara JM, Cassman KG. 1996: Effects of late nitrogen fertilizer application on head rice yield, protein content, and grain quality of rice. *Cereal Chem.*, 73, 556-560.

Rhodes D, Handa S, Bressan RA 1986: Metabolic changes associated with adaptation of plant cells to water stress. *Plant Physiol.* 82, 890-903.

Saikusa T, Horino T, Mori Y 1994a: Distribution of free amino acids in the rice and kernel fractions and the effect of water soaking on the distribution. *J.Agric.Food Chem.*, 42, 1122-1125.

Saikusa T, Horino T, Mori Y 1994b: Accumulation of γ-aminobutyric acid (Gaba) in the rice germ during water soaking. *Biosci.Biotech.Biochem.*, 58, 2291-2292.

Satya narayan V, Nair PM 1990: Metabolism, enzymology and possible roles of 4-aminobutyrate in higher plants. *Phytochem.*, 29, 367-375.

Snedden WA, Arazi T, Fromm H, Shelp BJ 1995: Calcium/Calmodulin activation of soybean glutamate decarboxylase. *Plant Physiol.*, 108, 543-549.

Taira H 1970: Effect of fertilization on protein content in high yield rice. *Jpn. J. Crop Sci.* 39: 200-203 (in Japanese with English summary).

Takebe M 1999: Improvement of the constituents related to crop quality by the control of nitrogen nutrition. *Bull. Nati. Agric. Res. Cent.*, 31, 19-83 (in Japanese with English summary).

Tsushida T, Murai T 1987: Conversion of glutamic acid to γ-aminobutyric acid in tea leaves under anaerobic conditions. *Agric. Biol.Chem.*, 51, 2865-2871.

Wallace W, Secor J, Schrader LE 1984: Rapid accumulation of γ-aminobutyric acid and alanine in soybean leaves in response to an abrupt transfer to lower temperature, darkness, or mechanical manipulation. *Plant Physiol.*, 75, 170-175.