Determination of γ -Aminobutyric Acid and Free Amino Acid Contents in Barley Seeds and Amounts Produced by Water Soaking Treatment

Yoichi NOGATA¹, Takashi YANAGISAWA², Daigo ABE¹, Takeshi SAITO¹, Atsuko YOSHIDA³, Hideaki OHTA³ and Takashi NAGAMINE^{4*}

¹ National Agricultural Research Center for Western Region, 1-3-1, Sen-yu, Zentsuji, Kagawa 765-8508, Japan

² National Institute of Crop Science, 2-1-18, Kan-nondai, Tsukuba, Ibaraki 305-8518, Japan

³ Graduate School of Health and Nutrition Sciences, Nakamura Gakuen University, 5-7-1 Befu, Johnan-ku, Fukuoka 814-0198, Japan

⁴ Hokuriku Research Center, National Agricultural Research Center, 1-2-1, Inada, Joetsu, Niigata 943-0193, Japan

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The contents of γ -aminobutyric acid (GABA) and free amino acid in barley seeds, and the amounts produced by water soaking treatment, were determined. In decreasing order among wholemeal, Kankei n553, Kankei n554, Hiproly, Yonkei 9551, and Beau Fiber contained GABA at 41.7, 27.1, 24.5, 21.9, and 19.3 mg/100 g, respectively, and all had high lysine (lys) endosperm mutations. Yonkei 9551 (25.6 mg/100 g) with high lys gene *lys1* and Yonkei 9552 (11.1 mg/100 g) with high lys gene *lys3.a* contained relatively large amounts of GABA in 60% flour. Barleys having high lys gene *lys3.a* contained a large amount of total free amino acid (338.2 – 626.2 mg/100 g) in wholemeal. The amino acids predominantly produced from the wholemeal of barley seeds generally matched those from wheat.

Keywords: barley, γ -aminobutyric acid, amino acid, bran, high lysine mutation

Introduction

Barley bran is recognized as a byproduct and is mainly used as feed for livestock. A number of approaches have been employed for its effective utilization as a source of biologically active compounds. Deguchi *et al.* (1999) focused on purple pigment, hordeumin, from barley bran-fermented broth and reported its antimutagenicity (Deguchi *et al.*, 2000a) and radical scavenging activity (Deguchi *et al.*, 2000b). Barley proanthocyanidins, abundant in bran, have been reported to potentiate retinoic acid-induced granulocytic and sodium butyrate-induced monocytic differentiation of HL60 cells (Tamagawa *et al.*, 1998), and have inhibitory effects on hyaluronidase activity, soybean lipoxygenase activity, and complementary activity (Tamagawa *et al.*, 1999). γ -Aminobutyric acid (GABA) is a non-protein amino acid and serves as a major transmitter inhibitor in mammalian systems (Nathan *et al.*, 1994). It has also been reported to have a reductive effect on blood pressure (Takahashi *et al.*, 1961), which has been attributed to a vascular dilatation ef-

fect (Billingsley *et al.*, 1980), and a suppressive effect on antidiuretic hormone secretion (Horn *et al.*, 1998). GABA is reportedly produced from rice bran or germ by the reaction of endogenous glutamate decarboxylase (GAD) and glutamate by water soaking treatment (Saikusa *et al.*, 1994). Since then, many attempts have been made to develop a method for its production from rice germ (Ohtsubo *et al.*, 2000), brown rice (Kinefuchi *et al.*, 1999), wheat germ (Takigawa *et al.*, 2009), and wheat bran (Nogata and Nagamine, 2009). In addition, fortification methods by fermentation have been reported (Yokoyama *et al.*, 2002; Kono and Himeno, 2000; Aoki *et al.*, 2003). With respect to the physiological effects of proteinaceous amino acids, leucine (Leu), glutamine (Gln), and arginine (Arg) have been reported to promote protein synthesis (Buse and Reid, 1975; Rennie *et al.*, 1986; Elman, 1988). Amino acid supplements including branched-chain amino acids (BCAA), Arg, and Gln resulted in quicker recovery from muscle fatigue following vigorous exercise training (Ohtani *et al.*, 2006). Proteinaceous amino acids have also been reported to be produced by the reaction of endogenous proteases from wheat bran (Nogata and Nagamine, 2009). From these reports, barley seeds and bran were expected to

*To whom correspondence should be addressed.

E-mail: naga@affrc.go.jp

produce GABA and free amino acids. However, knowledge of the content of GABA and free amino acids in barley seeds and their production levels by water soaking treatment is limited.

We, therefore, examined the GABA and free amino acid content in wholemeal and seed parts of barley samples and the amounts produced by water soaking treatment in order to estimate their levels and the ability of barley samples to produce them.

Materials and Methods

Materials Table 1 lists 20 selected cultivar and 20 breeding line barley samples, classified by two phenotypes, hulled or hull-less, and two-row or six-row, and high lys gene type. Among them, Daikei HQ10 (#15), Yonkei 9551 (#4), and Hiproly (#3) have the high lysine (lys) gene *lys1* mutation, Yonkei 9552 (#6), Norin PL2 (#21), and RISO 1508 (#8) have that of *lys3.a*, and Beau Fiber (#5), Kankei n552 (#7), Kankei n553 (#1), and Kankei n554 (#2) have that of *lys5.h*. They were grown and harvested in the fields of the Agricultural Research Center for Western Region, National Agriculture and Food Research Organization in Zentsuji, Kagawa, in 2009. Each cultivar or breeding line was grown with two replications and mixed together for experiments. For the experimental preparations, barley samples were cleaned of extraneous materials and their outer kernel lay-

ers were removed at a level of 40% weight in a grain testing mill (model TM-05, Satake, Hiroshima, Japan). The outer layer, inner particle, and whole grain samples were ground using a centrifugal mill (model ZM 200, Retsch, Germany) with a 0.5 mm screen at room temperature, and designated as bran, 60% flour, and wholemeal sample, respectively. Milled samples were kept at -30°C until use.

Water soaking treatment and determination of amino acids Portions (0.2 g) of the milled fractions were suspended in 4.0 mL of 50 mM sodium phosphate (pH 5.5) and reacted in a water bath with a wrist action shaker at 40°C for 1 h. After reaction, 4.0 mL of 16% trichloroacetic acid was added, the sample was centrifuged at $10,000 \times g$ for 20 min, and the supernatant was filtered through a 0.5 μm PTFE membrane filter followed by amino acid analysis. The quantification of GABA and α -amino acids was performed using a model L-8800 amino acid auto analyzer (Hitachi, Tokyo, Japan). Authentic amino acid standards were purchased from Wako Pure Chemicals (Tokyo, Japan). For each sample, the amount of produced amino acid was calculated by subtracting the blank value, which was presented as untreated value. All experiments were conducted in triplicate. Data are reported as mean $\pm$ standard deviation.

Results and Discussion

GABA content in wholemeal fraction The contents of GABA and total free α -amino acids in wholemeal samples were analyzed and are listed in Table 2. Large variation was observed in GABA content among tested samples. Cultivars with high GABA content were Hiproly (#3), Beau Fiber (#5), RISO 1508 (#8), and Harushirane (#10), with GABA concentrations of 24.5, 19.3, 17.9, and 15.3 mg/100 g, respectively. Among breeding lines and in decreasing order, Kankei n553 (#1), Kankei n554 (#2), Yonkei 9551 (#4), and Kankei n552 (#7) contained GABA at 41.7, 27.1, 21.9, and 17.9 mg/100 g, respectively. The reason for the large variation in GABA content is unknown at this stage; however, almost all barleys with high GABA content had high lys endosperm mutations: Yonkei 9551 (#4) and Hiproly (#3) had mutant gene *lys1*, RISO 1508 (#8) had *lys3.a*, and Beau Fiber (#5), Kankei n552 (#7), Kankei n553 (#1), and Kankei n554 (#2) had *lys5.h*. Mutations of *lys1* and *lys3.a* are accompanied by the over-expression of water-soluble high lys proteins (Munck and Wettstein, 1974). Mutations at the *lys5.h* locus are associated with a specific lesion in the transport of ADP-glucose across the plastid membrane (Patron *et al.*, 2004). In breeding, this mutant is used as source material to develop barley cultivars high in β -glucan. In plants, GABA is produced in the cytoplasm through the decarboxylation of glutamate catalyzed by GAD (Bown and Shelp, 1997). In order to elucidate the

Table 1. Barley samples investigated in this study.

Sample #	Sample name	Cultivar or breeding line	Source	Hulled or hull-less	Two-row or six-row	Gene (high lys)
1	Kankei n553	Breeding line	Japan (Ibaraki)	Hull-less	six	<i>lys5.h</i>
2	Kankei n554	Breeding line	Japan (Ibaraki)	Hull-less	six	<i>lys5.h</i>
3	Hiproly	Cultivar	Ethiopia	Hull-less	two	<i>lys1</i>
4	Yonkei 9551	Breeding line	Japan (Kagawa)	Hull-less	two	<i>lys1</i>
5	Beau Fiber	Cultivar	Japan (Ibaraki)	Hull-less	two	<i>lys5.h</i>
6	Yonkei 9552	Breeding line	Japan (Kagawa)	Hull-less	two	<i>lys3.a</i>
7	Kankei n552	Breeding line	Japan (Ibaraki)	Hull-less	six	<i>lys5.h</i>
8	Riso 1508	Cultivar	Denmark	Hulled	two	<i>lys3.a</i>
9	America mochimugi	Breeding line	USA	Hulled	two	
10	Harushirane	Cultivar	Japan (Ibaraki)	Hulled	six	
11	Elrose	Cultivar	Canada	Hulled	two	
12	Sukai golden	Cultivar	Japan (Tochigi)	Hulled	two	
13	Shikoku hadaka mochi 113	Breeding line	Japan (Kagawa)	Hull-less	six	
14	Daikei HL9-2-6	Breeding line	Japan (Tochigi)	Hulled	two	
15	Daikei HQ10	Breeding line	Japan (Tochigi)	Hulled	two	<i>lys1</i>
16	Stirling	Cultivar	Australia	Hulled	two	
17	Mannenboshi	Cultivar	Japan (Kagawa)	Hull-less	six	
18	Scarlett	Cultivar	Canada	Hulled	two	
19	BF 99-1	Breeding line	Japan (Tochigi)	Hulled	two	
20	Shikoku hadaka mochi 107	Breeding line	Japan (Kagawa)	Hull-less	two	
21	Norin PL2	Cultivar	Japan (Kagawa)	Hull-less	two	<i>lys3.a</i>
22	M-737	Cultivar	Canada	Hulled	two	
23	Shikoku hadaka 84	Breeding line	Japan (Kagawa)	Hull-less	two	
24	Tochinoibuki	Cultivar	Japan (Tochigi)	Hulled	two	
25	Tozan kawa 107	Breeding line	Japan (Nagano)	Hulled	six	
26	Yon R kei 2815	Breeding line	Japan (Kagawa)	Hulled	two	
27	Kanto kawa 87	Breeding line	Japan (Ibaraki)	Hulled	six	
28	Pirkka	Cultivar	Finland	Hulled	six	
29	Nepal hadaka 1372 (H)	Breeding line	Nepal	Hull-less	six	
30	Yonezawa mochi 2	Cultivar	Japan (Mie)	Hull-less	six	
31	Ethiopia 84	Breeding line	Ethiopia	Hulled	six	
32	Daishi mochi	Cultivar	Japan (Kagawa)	Hull-less	six	
33	Ethiopia 506	Breeding line	Ethiopia	Hulled	six	
34	Ichibanboshi	Cultivar	Japan (Kagawa)	Hull-less	six	
35	Canada 1602	Cultivar	Canada	Hulled	six	
36	Kanto niyo 40	Breeding line	Japan (Tochigi)	Hulled	two	
37	Atlas 46	Cultivar	USA	Hulled	six	
38	Nepal hadaka 1368 (H)	Breeding line	Nepal	Hull-less	six	
39	Glacier AC38	Cultivar	USA	Hulled	six	
40	Glacier	Cultivar	USA	Hulled	six	

Table 2. GABA and total free α -amino acid contents in the wholemeal of barley samples.

Sample #	Sample name	GABA (mg/100 g)	Total free α -amino acids (mg/100 g)
1	Kankei n553	41.7 $\pm$ 3.8	252.3 $\pm$ 27.0
2	Kankei n554	27.1 $\pm$ 2.3	209.5 $\pm$ 21.1
3	Hiproly	24.5 $\pm$ 2.1	243.7 $\pm$ 26.9
4	Yonkei 9551	21.9 $\pm$ 1.9	141.7 $\pm$ 15.3
5	Beau Fiber	19.3 $\pm$ 2.1	183.0 $\pm$ 19.9
6	Yonkei 9552	18.2 $\pm$ 1.8	395.5 $\pm$ 43.8
7	Kankei n552	17.9 $\pm$ 1.9	191.0 $\pm$ 20.6
8	Riso 1508	17.9 $\pm$ 1.6	338.3 $\pm$ 36.2
9	America mochimugi	15.4 $\pm$ 1.6	200.2 $\pm$ 21.6
10	Harushirane	15.3 $\pm$ 1.7	167.5 $\pm$ 17.8
11	Elrose	13.7 $\pm$ 1.2	143.7 $\pm$ 15.9
12	Sukai golden	11.9 $\pm$ 1.3	168.4 $\pm$ 17.2
13	Shikoku hadaka mochi 113	10.8 $\pm$ 1.2	206.6 $\pm$ 21.0
14	Daikei HL9-2-6	10.4 $\pm$ 1.4	107.2 $\pm$ 11.1
15	Daikei HQ10	9.7 $\pm$ 1.2	183.2 $\pm$ 19.5
16	Stirling	9.5 $\pm$ 1.1	135.8 $\pm$ 14.3
17	Mannenboshi	9.2 $\pm$ 1.0	166.4 $\pm$ 18.5
18	Scarlett	9.0 $\pm$ 0.9	154.8 $\pm$ 16.6
19	BF 99-1	8.4 $\pm$ 1.0	116.6 $\pm$ 12.8
20	Shikoku hadaka mochi 107	8.3 $\pm$ 0.8	75.8 $\pm$ 6.9
21	Norin PL2	8.0 $\pm$ 0.9	626.2 $\pm$ 67.1
22	M-737	7.9 $\pm$ 0.9	300.4 $\pm$ 34.6
23	Shikokuhadaka 84	7.7 $\pm$ 0.8	120.0 $\pm$ 13.8
24	Tochinoibuki	7.5 $\pm$ 0.9	119.4 $\pm$ 13.3
25	Tozan kawa 107	7.3 $\pm$ 0.9	69.2 $\pm$ 7.5
26	Yon R kei 2815	7.3 $\pm$ 0.8	86.6 $\pm$ 9.6
27	Kanto kawa 87	7.1 $\pm$ 0.7	90.8 $\pm$ 9.7
28	Pirkka	6.3 $\pm$ 0.7	108.0 $\pm$ 10.7
29	Nepal hadaka 1372 (H)	6.2 $\pm$ 0.6	95.5 $\pm$ 10.3
30	Yonezawa mochi 2	6.1 $\pm$ 0.7	91.9 $\pm$ 10.7
31	Ethiopia 84	5.6 $\pm$ 0.5	175.7 $\pm$ 18.8
32	Daishi mochi	5.4 $\pm$ 0.6	93.1 $\pm$ 10.6
33	Ethiopia 506	5.3 $\pm$ 0.4	141.2 $\pm$ 15.0
34	Ichibanboshi	5.2 $\pm$ 0.6	117.3 $\pm$ 12.1
35	Canada 1602	4.7 $\pm$ 0.5	181.2 $\pm$ 19.2
36	Kanto nijo 40	4.5 $\pm$ 0.6	114.9 $\pm$ 12.2
37	Atlas 46	4.4 $\pm$ 0.3	106.0 $\pm$ 12.7
38	Nepal hadaka 1368 (H)	4.3 $\pm$ 0.5	105.5 $\pm$ 11.1
39	Glacier AC38	3.8 $\pm$ 0.4	162.8 $\pm$ 17.9
40	Glacier	2.7 $\pm$ 0.3	122.8 $\pm$ 14.6

Data are expressed as the mean $\pm$ standard deviation of three replicates.

relationship between lys mutation and high GABA content, studies on the supply of glutamate, calcium, and calmodulin, as well as the expression of GAD, are considered necessary. The GABA content in brown rice (Koshihikari) was reported to be 3.8–6.4 mg/100 g (Saikusa *et al.*, 1994; Kinefuchi *et al.*, 1999), which was lower than the average of barley samples without lys mutation (7.7 mg/100 g).

Total free amino acid content in wholemeal fraction

Contents of total free α -amino acids in samples varied as for the GABA result (Table 2). Cultivars containing a large amount of amino acids were Norin PL2 (#21), RISO 1508 (#8), M-737 (#22), and Hiproly (#3), with 626.2, 338.3, 300.4, and 243.7 mg/100 g, respectively. Among breeding lines, Yonkei 9552 (#6), Kankei n553 (#1), and Kankei n554 (#2) contained total amino acids at 395.5, 252.3, and 209.5 mg/100 g, respectively. Although M-737 (#22) had no mutation but was a low β -glucan and hordein line, other barleys had some type of high lys endosperm mutation. In particular, all three barleys with the largest amino acid content had high lys gene *lys3.a*. In the case of barleys with *lys5.h* mutation,

total amino acid content ranged from 183.0 to 252.3 mg/100 g, indicating its moderate effect. From this result, *lys3.a* was shown to play a role in the fortification of free amino acids in barley seeds, although its mechanism is not clear. Tozan kawa 107 (#25) contained the smallest amount of total free amino acids among tested samples, 69.2 mg/100 g, which was about the same level as brown rice (Koshihikari, 63.1 – 67 mg/100 g) (Saikusa *et al.*, 1994; Kinefuchi *et al.*, 1999). There was no significant difference between hulled and hull-less, or two-row and six-row barleys in terms of the contents of GABA and total amino acids.

GABA content in milled fractions of barley seeds Figure 1 shows the GABA content in milled fractions of selected samples and the amount released by water soaking treatment for 1 h. In untreated samples, GABA was generally present at higher levels in bran than other fractions (Fig. 1 (B)). Remarkably, barleys containing a relatively large amount of

Fig. 1. GABA content in milled fractions of selected barley seeds and amount produced by water soaking treatment. ■: Contents in untreated sample, □: amounts produced after water soaking treatment.

GABA in the food part, such as Yonkei 9551 (25.6 mg/100 g) and Yonkei 9552 (11.1 mg/100 g), were found (Fig. 1 (C)). Interestingly, Yonkei 9551 contained a larger amount of GABA in 60% flour than in the bran fraction (22.5 mg/100 g). Since this trait has not been reported for other crops, screening and breeding of barleys with high GABA in the endosperm is expected to enhance their value. The release of GABA by water soaking treatment from the 60% flour fraction of Yonkei 9551 was virtually zero, while glutamic acid (Glu) was contained at 22.6 mg/100 g (data not listed). This result suggested the lack of GAD in the mature endosperm and that the GABA synthesis occurred during development. On the other hand, GABA synthesis is known to occur in response to conditions of environmental stress (Bouche and Fromm, 2004). GAD is activated by stress-induced increases in calcium and has an optimum pH similar to those occurring in response to stress conditions (Arazi *et al.*, 1995). In order to clarify the reason for the enrichment of GABA in the endosperm of barleys, the expression and distribution of GAD, and concentrations of calcium and calmodulin (Johnson *et al.*, 1997) during development of seeds should be studied. Specific foods containing GABA have been granted "Foods for Specified Health Uses" (FOSHU) status owing to the physiological function of GABA on blood pressure. For FOSHU containing GABA, its intake is defined as 20 mg a day. Taking this much GABA from staple crops is considered beneficial because the daily consumption of these crops is large and is a natural form of nutrient intake.

Production of GABA by water soaking treatment With respect to the GABA production by water soaking treatment, the bran fraction produced a larger amount than other fractions, while the produced amount from 60% flour fractions was low or zero (Fig. 1 (A), (B), and (C)). In the bran fraction, Norin PL2 and Yonkei 9552, both having *lys3.a* mutation, produced relatively more GABA than the others, 31.5 and 19.3 mg/100 g, respectively. To date, the reason for the difference in GABA-producing ability is not clear. As shown in Table 2, *lys3.a* mutants such as Norin PL2 (#21), Yonkei 9552 (#6), and RISO 1508 (#8) contained a larger amount of total free amino acids than other samples. One reason for the higher ability of Norin PL2 and Yonkei 9552 to produce GABA may be that they contain a large amount of Glu, which can be converted to GABA. Methods for producing GABA by soaking materials in a Glu solution have been reported (Takigawa *et al.*, 2009; Ohtsubo *et al.*, 2000). These methods can be applied to barley seed parts, although detailed examination is necessary.

Correlation between initial Glu content and produced amount of GABA Figure 2 shows the correlation between initial Glu content and amount of GABA produced by wa-

ter soaking treatment for 1 h in wholemeal (Fig. 2 (A)) and bran (Fig. 2 (B)) fractions. Strong correlations between the content of Glu and produced level of GABA: $R = 0.977$ for wholemeal and $R = 0.976$ for the bran fraction ($P < 0.01$ for both fractions) were observed. These results indicate that the amount of GABA produced by water soaking treatment for 1 h does not depend on the property of a sample but its initial content of Glu. The Glu contents after 1 h of treatment for Norin PL2 and Yonkei 9552 were 44.8 and 36.4 mg/100 g for the wholemeal fraction (Table 3), and 93.5 and 61.7 mg/100 g for the bran fraction (data not listed), respectively. Thus, the extension of reaction time probably causes increased GABA production.

Total free amino acid content in milled fractions of barley seeds The total free α -amino acid content and the amount released by water soaking treatment from milled fractions of barley seeds are shown in Fig. 3 (A), (B), and (C). The total contents in each fraction of Norin PL2 and Yonkei 9552 were exceptionally large among tested samples. Unlike GABA content, all samples commonly contained total amino acids in decreasing order of bran, wholemeal, and 60% flour. The reason for these two barleys having a large content is not

Fig. 2. Correlations between initial Glu content and amount of GABA produced by water soaking treatment. (A): Wholemeal fraction. (B): Bran fraction. Yonkei 9551 (1), Yonkei 9552 (2), Norin PL2 (3), Shikokuhadaka 84 (4), Mannenboshi (5), Daishimochi (6), Ichibanboshi (7).

Table 3. Initial content and amount (mg/100 g) of α -amino acids produced by water soaking treatment of wholemeal fraction.

	Yonkei 9551		Yonkei 9552		Norin PL2		Shikokuhadaka 84		Mannenboshi		Daishi mochi		Ichibanboshi	
	initial	produced	initial	produced	initial	produced	initial	produced	initial	produced	initial	produced	initial	produced
Aliphatic amino acids														
Gly	4.8 $\pm$ 0.5	0.6 $\pm$ 0.1	9.8 $\pm$ 1.2	1.7 $\pm$ 0.2	9.0 $\pm$ 1.0	1.4 $\pm$ 0.2	4.4 $\pm$ 0.4	0.8 $\pm$ 0.1	4.4 $\pm$ 0.5	0.7 $\pm$ 0.1	2.7 $\pm$ 0.3	1.0 $\pm$ 0.1	2.4 $\pm$ 0.2	0.9 $\pm$ 0.2
Ala	14.5 $\pm$ 1.6	1.0 $\pm$ 0.2	31.0 $\pm$ 3.6	2.2 $\pm$ 0.3	28.0 $\pm$ 3.1	2.1 $\pm$ 0.3	10.9 $\pm$ 1.3	2.0 $\pm$ 0.3	17.3 $\pm$ 1.9	1.7 $\pm$ 0.2	5.5 $\pm$ 0.8	2.8 $\pm$ 0.3	6.0 $\pm$ 0.9	2.5 $\pm$ 0.3
Val	7.3 $\pm$ 0.6	1.3 $\pm$ 0.2	20.9 $\pm$ 2.5	5.7 $\pm$ 0.6	25.8 $\pm$ 2.5	5.1 $\pm$ 0.6	3.7 $\pm$ 0.3	2.1 $\pm$ 0.3	6.2 $\pm$ 0.7	1.9 $\pm$ 0.2	1.7 $\pm$ 0.3	2.8 $\pm$ 0.4	2.8 $\pm$ 0.4	2.5 $\pm$ 0.3
Ile	3.4 $\pm$ 0.3	1.0 $\pm$ 0.1	11.2 $\pm$ 2.0	3.6 $\pm$ 0.4	12.9 $\pm$ 1.4	2.9 $\pm$ 0.6	2.0 $\pm$ 0.2	1.4 $\pm$ 0.1	2.5 $\pm$ 0.3	1.0 $\pm$ 0.2	0.7 $\pm$ 0.1	1.9 $\pm$ 0.2	1.2 $\pm$ 0.1	1.5 $\pm$ 0.2
Leu	4.1 $\pm$ 0.3	3.2 $\pm$ 0.3	13.1 $\pm$ 1.7	9.1 $\pm$ 0.7	16.1 $\pm$ 1.3	7.7 $\pm$ 0.8	2.6 $\pm$ 0.2	4.2 $\pm$ 0.3	3.3 $\pm$ 0.4	3.2 $\pm$ 0.5	0.8 $\pm$ 0.0	5.3 $\pm$ 0.3	1.8 $\pm$ 0.1	4.6 $\pm$ 0.6
Dicarboxylic amino acids and amides														
Asp	17.6 $\pm$ 2.2	-1.2 $\pm$ 0.4	22.4 $\pm$ 2.9	-0.8 $\pm$ 0.1	63.8 $\pm$ 8.2	tr	13.6 $\pm$ 1.6	-1.2 $\pm$ 0.2	18.2 $\pm$ 2.0	tr	14.2 $\pm$ 2.0	-1.5 $\pm$ 0.2	17.7 $\pm$ 2.1	-0.4 $\pm$ 0.1
Glu	23.4 $\pm$ 2.6	-3.7 $\pm$ 0.5	41.8 $\pm$ 5.4	-5.4 $\pm$ 0.7	68.4 $\pm$ 10.1	-23.6 $\pm$ 2.9	15.2 $\pm$ 2.0	-2.4 $\pm$ 0.4	27.5 $\pm$ 3.2	-8.6 $\pm$ 1.3	13.9 $\pm$ 1.9	3.9 $\pm$ 0.4	20.3 $\pm$ 2.4	-3.2 $\pm$ 0.4
Asn	25.5 $\pm$ 3.1	0.7 $\pm$ 0.1	64.7 $\pm$ 7.9	2.4 $\pm$ 0.4	122.4 $\pm$ 28.7	-2.3 $\pm$ 0.6	30.4 $\pm$ 4.1	0.7 $\pm$ 0.1	28.1 $\pm$ 3.6	0.8 $\pm$ 0.1	31.3 $\pm$ 4.2	2.0 $\pm$ 0.3	30.0 $\pm$ 3.8	tr
Gln	5.1 $\pm$ 0.6	0.9 $\pm$ 0.1	15.7 $\pm$ 2.1	2.5 $\pm$ 0.2	26.2 $\pm$ 3.5	2.0 $\pm$ 0.2	1.8 $\pm$ 0.1	0.8 $\pm$ 0.1	5.3 $\pm$ 0.6	0.8 $\pm$ 0.1	tr	2.2 $\pm$ 0.2	0.5 $\pm$ 0.1	1.7 $\pm$ 0.3
Basic amino acids														
Arg	5.4 $\pm$ 0.3	3.1 $\pm$ 0.2	23.8 $\pm$ 3.6	9.0 $\pm$ 0.8	51.8 $\pm$ 3.9	4.0 $\pm$ 0.4	6.3 $\pm$ 0.4	5.0 $\pm$ 0.3	8.7 $\pm$ 0.8	4.0 $\pm$ 0.3	5.5 $\pm$ 0.4	6.2 $\pm$ 0.4	4.4 $\pm$ 0.2	5.1 $\pm$ 0.3
Lys	4.8 $\pm$ 0.2	3.2 $\pm$ 0.1	17.9 $\pm$ 2.7	9.3 $\pm$ 0.6	24.9 $\pm$ 1.6	6.6 $\pm$ 0.3	4.1 $\pm$ 0.2	3.7 $\pm$ 0.2	3.9 $\pm$ 0.2	3.2 $\pm$ 0.2	2.5 $\pm$ 0.2	4.9 $\pm$ 0.3	3.6 $\pm$ 0.2	3.4 $\pm$ 0.2
His	2.7 $\pm$ 0.1	0.9 $\pm$ 0.0	8.6 $\pm$ 0.9	1.8 $\pm$ 0.1	20.8 $\pm$ 1.4	tr	2.4 $\pm$ 0.1	1.0 $\pm$ 0.2	5.2 $\pm$ 0.2	0.9 $\pm$ 0.1	2.2 $\pm$ 0.1	0.8 $\pm$ 0.1	3.2 $\pm$ 0.2	0.6 $\pm$ 0.1
Aromatic amino acids														
Trp	1.4 $\pm$ 0.1	0.9 $\pm$ 0.2	7.5 $\pm$ 0.5	tr	11.4 $\pm$ 0.8	4.0 $\pm$ 0.9	5.7 $\pm$ 0.8	6.4 $\pm$ 0.6	13.1 $\pm$ 1.0	9.8 $\pm$ 1.1	0.8 $\pm$ 0.0	1.7 $\pm$ 0.1	5.0 $\pm$ 0.4	4.0 $\pm$ 0.3
Phe	3.2 $\pm$ 0.2	2.5 $\pm$ 0.2	12.9 $\pm$ 0.9	6.8 $\pm$ 0.5	21.8 $\pm$ 1.3	6.3 $\pm$ 0.4	2.2 $\pm$ 0.2	2.9 $\pm$ 0.3	3.3 $\pm$ 0.2	2.1 $\pm$ 0.2	1.1 $\pm$ 0.1	3.4 $\pm$ 0.2	2.6 $\pm$ 0.2	2.9 $\pm$ 0.3
Tyr	4.5 $\pm$ 0.2	1.9 $\pm$ 0.1	14.1 $\pm$ 1.3	5.3 $\pm$ 0.2	19.0 $\pm$ 2.0	4.2 $\pm$ 0.2	3.1 $\pm$ 0.2	2.3 $\pm$ 0.2	3.1 $\pm$ 0.2	1.9 $\pm$ 0.1	1.5 $\pm$ 0.1	2.3 $\pm$ 0.2	3.0 $\pm$ 0.3	2.3 $\pm$ 0.2
Sulfur, hydroxyl, and cyclic amino acids														
Met	1.3 $\pm$ 0.1	1.2 $\pm$ 0.1	2.5 $\pm$ 0.3	3.9 $\pm$ 0.3	1.8 $\pm$ 0.2	3.5 $\pm$ 0.4	0.5 $\pm$ 0.0	1.5 $\pm$ 0.1	1.1 $\pm$ 0.1	1.4 $\pm$ 0.1	tr	2.0 $\pm$ 0.3	tr	1.9 $\pm$ 0.2
Ser	4.0 $\pm$ 0.2	1.1 $\pm$ 0.1	12.9 $\pm$ 1.8	3.2 $\pm$ 0.2	15.8 $\pm$ 2.4	2.6 $\pm$ 0.1	2.2 $\pm$ 0.1	1.0 $\pm$ 0.1	4.6 $\pm$ 0.3	1.2 $\pm$ 0.1	0.8 $\pm$ 0.0	1.9 $\pm$ 0.2	1.8 $\pm$ 0.1	1.5 $\pm$ 0.2
Thr	3.7 $\pm$ 0.2	0.9 $\pm$ 0.1	8.3 $\pm$ 0.6	3.2 $\pm$ 0.2	14.2 $\pm$ 1.7	1.8 $\pm$ 0.1	2.3 $\pm$ 0.2	1.0 $\pm$ 0.1	3.4 $\pm$ 0.2	0.9 $\pm$ 0.0	0.9 $\pm$ 0.1	1.6 $\pm$ 0.2	1.4 $\pm$ 0.1	1.6 $\pm$ 0.2
Pro	4.8 $\pm$ 0.5	tr	54.4 $\pm$ 6.3	-1.6 $\pm$ 0.0	65.9 $\pm$ 7.0	2.7 $\pm$ 0.1	4.8 $\pm$ 0.4	tr	9.7 $\pm$ 1.0	-2.9 $\pm$ 0.2	6.5 $\pm$ 0.7	0.7 $\pm$ 0.0	4.2 $\pm$ 0.4	tr
Total	141.7 $\pm$ 15.5	19.3 $\pm$ 2.4	395.5 $\pm$ 43.6	60.3 $\pm$ 7.5	626.2 $\pm$ 73.6	26.6 $\pm$ 3.1	119.9 $\pm$ 13.5	33.0 $\pm$ 3.8	169.2 $\pm$ 18.7	23.8 $\pm$ 3.3	93.1 $\pm$ 10.4	45.6 $\pm$ 6.7	111.8 $\pm$ 13.1	34.2 $\pm$ 4.8

Data are expressed as the mean $\pm$ standard deviation of three replicates.

tr: detected but too small to quantify.

Fig. 3. Total free amino acid content in milled fractions of selected barley seeds and amount produced by water soaking treatment. ■: Contents in untreated sample, □: amount produced after water soaking treatment.

clear but may have some relation with their *lys3.a* mutation. On the other hand, Yonkei 9551, having the high *lys* allele *lys1*, contained total amino acids at the same level as normal-type barleys in all tested fractions. The difference between these two mutations may be associated with different abilities to convert amino acids to proteins.

Production of free amino acids by water soaking treatment No specific relationship between amino acid-producing activity and high *lys* mutations (Fig. 3 (B)) was evident. The rate of produced amount against the initial content of total amino acids was apparently lower than that of GABA for both wholemeal and bran. Concerning the amount produced from the wholemeal fraction, the largest value was 60.3 mg/100 g of Yonkei 9552 followed by 45.6 mg/100 g of Daishimochi and 34.2 mg/100 g of Ichibanboshi. In the case of wheat wholemeal, the average produced amounts under the same conditions were 72.4 and 65.6 mg/100 g for soft and hard wheat, respectively (Nogata and Nagamine, 2009). The amount produced from the bran fraction of Fukusayaka wheat, with one of the lowest amino acid-producing abilities among tested samples, was 82.5 mg/100 g (Nogata and Nagamine, 2009), while the largest produced among tested barley samples was from Yonkei 9551, at 82.1 mg/100 g. Therefore, the ability to produce free amino acids by water soaking treatment of barley seeds was considered rather weak compared with that of wheat.

Free amino acid content and amount produced by water soaking treatment Individual free α -amino acids in wholemeal fractions and amount produced by water soaking treatment were analyzed. In terms of the initial level of amino

acids, the contents of dicarboxylic amino acid and amide, such as aspartic acid (Asp), Glu, and asparagine (Asn), were found to be high in all samples (Table 3). Yonkei 9552 and Norin PL2 having *lys3.a* mutation contained a larger amount of each amino acid except tryptophan (Trp). In terms of the amino acid composition on the basis of molecular weight, these barleys contained higher proportions of proline (Pro), valine (Val), arginine (Arg), lysine (Lys), glutamine (Gln), leucine (Leu), isoleucine (Ile), phenylalanine (Phe), and tyrosine (Tyr) than normal-type barleys. The proportions of Pro, Val, Arg, Lys, Gln, Leu, Ile, Phe, and Tyr were 15.3, 5.8, 4.4, 4.0, 3.5, 3.2, 2.8, 2.5, and 2.5% for Yonkei 9551, and 12.2, 4.2, 6.4, 3.6, 3.8, 2.6, 2.1, 2.8, and 2.2% for Norin PL2, respectively (data not listed). Inversely, the proportions of Asn, Glu, Asp, and glycine of normal-type barleys were higher than those with high lys mutation (data not listed). In the amino acid composition of Yonkei 9551 having *lys1* mutation, the proportions of Val, Ile, Leu, Gln, and Tyr were higher than those of normal-type barleys, which partly matched those with *lys3.a* mutation. From these results, high lys mutation was suggested to affect both free amino acid content and composition in barley seeds.

The content of Glu was reduced by water soaking treatment in accordance with the production of GABA, with the exception of Daishimochi, which produced only 3.0 mg/100 g of GABA. Among amino acids produced by water soaking treatment, the aliphatic amino acids Val and Leu, basic amino acids Arg and Lys, and aromatic amino acids Phe and Tyr were produced at higher levels than the others. Similarly, the wholemeal fraction of wheat seeds was reported to produce Val, Leu, Arg, Lys, Trp, and Pro at high levels under the same conditions (Nogata and Nagamine, 2009). The similarity of amino acids produced at high levels from barley and wheat seeds may be attributed to hydrolysis of storage proteins with similar amino acid compositions.

In this study, we analyzed GABA and free amino acid contents in barley seeds and the amounts produced by water soaking treatment. As a result, barleys with high GABA content in the endosperm and those with high free amino acid content in each seed part were discovered, and effective production of amino acids by water soaking treatment was confirmed. These findings are expected to be utilized in the field of breeding and at the food processing stage. On the other hand, the relationship between GABA and free amino acid content and high lys mutation types is unknown. Further studies are needed to elucidate these subjects.

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