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Physicochemical Properties of a Giant Embryo Mutant Induced by T-DNA Insertion in Rice

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ABSTRACT This study was conducted to determine the physicochemical properties of a giant embryo rice 'P47JB-4-B-5-B' derived from the cross between 'P47', a mutant of 'Hwayoung' induced by T-DNA insertion, and 'Junam'. The grain appearance and chemical components of the embryo were analyzed and compared with a donor cultivar, 'Hwayoung'. The proportion of embryo weight to grain weight of 'P47 JB-4-B-5-B' was 2.2 times heavier (6.7%) than that (3.1%) of 'Hwayoung'. Total free amino acid content (75.81 mg/100 g) of 'P47JB-4-B-5-B' was 2.1 times higher than that of 'Hwayoung'. The GABA content in brown rice was 14.06 mg/100 g in 'P47JB-4-B-5-B' and 6.8 mg/100 g in 'Hwayoung'. Especially, the GABA content in brown rice of 'P47JB-4-B-5-B' remarkably increased (about 33 times from 1.48 mg to 44.81 mg/100 g) 2 days after germination. Continuous frequency distributions and transgressive segregation in embryo length and width were observed in the F_2 population of the cross between 'P47' and 'Cheongcheong', indicating that the giant embryo was controlled by quantitative trait loci. However, embryo length and width demonstrated high broad sense heritability, implying that giant embryonic traits could be selected in earlier generations in comparison with other quantitative traits.

Keywords : rice, giant embryo, T-DNA insertion

Rice is a major cereal feed in half of the world population. In Asia, more than 2 billion people obtain their daily calorie intake from rice. Especially in developing countries, about 27% of daily energy and 20% of daily protein are obtained from rice. In the 1970s, self-supporting rice was produced by breeding of semi-dwarf and heavy panicle 'Tongil' type rice cultivar in Korea. However, rice consumption was largely reduced in the 2000s due to the increasing economy and westernized dietary habits. Thus, the development of a functional rice cultivar with high quality to meet consumer demand has become very important.

Brown rice is not ordinarily accepted by consumers due to its dark color and hard taste. Nevertheless, it contains abundant protein content, dietary fiber, phytic acid, vitamins, GABA (gamma amino butyric acid), and other functional nutrients. Especially, GABA is a representative depressive neurotransmitter that participates in the sympathetic nervous system, and it has been proven effective in lowering blood pressure in animal models and humans. Moreover, GABA-enriched foods can be used as a dietary supplement and nutraceutical for the treatment of sleeplessness, depression, autonomic disorders, chronic alcohol-related symptoms, and for the stimulation of immune cells (Zhang *et al.*, 2006). Generally speaking, the human body can produce a sufficient amount of GABA, but poor diet, little or no physical activity, overindulgence of estrogen, salicylate, and food additives, deficiency of zinc and vitamin B, stress, toxic agents in the environment, and so on can all adversely affect GABA production (Kang *et al.*, 2007).

Even though the embryo contains only a small proportion of nutrients as compared to whole brown rice (about 2-3%), it contains abundant proteins and vitamins as compared to the endosperm (Hinton, 1948; Juliano, 1985). It is noteworthy that giant embryo contains much more fatty acids, amino acids, vitamins, crude protein content, and GABA content than common rice embryo (Nagasawa, 1996; Zhang *et al.*, 2005; Choi *et al.*, 2006). In addition, GABA content can reach its highest level after germination of brown rice (Saikusa *et al.*, 1994; Choi *et al.*, 2006).

Satoh *et al.* (1981) and Hong *et al.* (1995) selected giant

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embryonic lines treated with ethyl methanesulfonate (EMS) and N-methyl-N-nitrosourea (MNU) and implemented giant embryonic gene mapping. Jeon *et al.* (2000) developed giant embryonic lines by T-DNA insertion. Satoh *et al.* (1990) reported that the gene related to giant embryo is located on chromosome 7. Later, Koh *et al.* (1996) mapped the ge^s gene controlling giant embryo on chromosome 7 with flanking markers RZ395 and CDO497, and a similar location for ge^s was also reported by Qian *et al.* (1996).

In the present study, giant embryonic mutants were developed from a donor cultivar, 'Hwayoung', by T-DNA insertion. The embryo size of the giant embryonic lines was 2-3 times that of 'Hwayoung'. However, disruption of chromosome balance due to T-DNA insertion led to relatively poor agronomic traits and grain appearance. To overcome this flaw, 'Junam' with high yield and good grain appearance was used as a recurrent parent in crosses with the giant embryonic lines. Thus, a candidate line, 'P47JB-4-B-5-B', with good agronomic traits, brown rice and embryonic properties, and physicochemical characteristics was selected and analyzed in comparison with the donor cultivar 'Hwayoung'.

MATERIALS AND METHODS

Plant materials

The selected giant embryonic line 'P47JB-4-B-5-B' obtained from a backcross of 'P47' (a giant mutant line from POS TECH in 2005) and recurrent parent 'Junam', donor cultivar 'Hwayoung', for comparison purposes were used in this study. 'P47' is a mutant line derived from 'Hwayoung' by T-DNA insertion, whereas 'Junam' is a high yield-high quality cultivar. Due to the poor agronomic traits and grain appearance of 'P47', a backcross population (BC_2) of 'P47' and 'Junam' was constructed with the aim of breeding a giant embryonic line with good agronomic and grain traits. Therefore, 'P47JB-4-B-5-B' was selected by the bulk and pedigree breeding method. Experimental trials were carried out at the experimental plots of Kyungpook National University (KNU) in Gunwi from 2007 to 2010. Seedling and transplantation were carried out on 30 April and 28 May, respectively. One plant per hill was transplanted with spaces of 30 cm between lines and 15 cm between plants. The management and fertilizer application followed $N\text{-}P_2O_5\text{-}K_2$=9-4.5-5.7/10a.

Evaluation for characteristics

The grain appearance of brown rice were examined after 50 days of heading and naturally dried until 15% of water content remained. Grain length, width, embryo length, and width were examined for 30 grains per line or cultivar using a vernier calipers and 50* microscope. The 1000-grain weight and 100-embryo weight were determined for three replications.

Analysis for physicochemical components

Free amino acid, free sugar, and fatty acid contents were analyzed at the physiology lab, NICS and Local innovation center of KNU. For free amino acid content, 0.2 g of sample was put into 15 ml tube and agitated after adding 1 ml of 99.9% ethanol and 1 ml of 4% sulfosalycylic acid, followed by degradation with high frequency on ice for 10 min. The mixture was then centrifuged at 3000 rpm for 15 min, after which the upper layer was filtered through a 0.2 μm syringe filter (GHP). An AccQ.Tag Derivatization kit was used to induce amino acids in the samples, followed by analysis with a Waters-UPLC (AccQ.Taq Ultra column).

For free sugar content, 0.5 g of rice powder from each sample was agitated with 20 ml of ddH_2O, filtered, and then centrifuged at 5000 rpm for 15 min. The suspension was then filtered through a 0.45 μm Millipore filter and Sep-Pak NH_2 column. A Supelco LC-NH_2 column was used to remove pigment and impurity matter, followed by analysis by HPLC.

For fatty acid content, the methanol : heptane : benzene : 2,2-dimethoxypropane : H_2SO_4 (37:36:20:5:2, v/v) was added to 300 mg of rice powder from each sample. After heating at 80 degrees, the single phase was chilled at room temperature. Then, the suspension consisting of fatty acid methyl esters (FAMEs) was analyzed by capillary GC.

In order to determine the GABA content and free sugar contents of germinated brown rice, 20 g of brown rice was induced to germinate at 30 degrees in Petri dishes after soaking with ddH_2O for 3 hours. To avoid seed dryness, water was added every 4 hours during 24 hours and 48 hours of germination. The sample was then lyophilized and grinded for filtration through 100-mesh.

Genetic analysis for giant embryo

The embryo length and width of the F_2 population, which consisted of 220 plants derived from the cross between ‘P47’ and ‘Cheongcheong’, were measured to evaluate broad sense habitability.

RESULTS AND DISCUSSION

Grain appearance of ‘P47JB-4-B-5-B’

In comparison with ‘Hwayoung’ and ‘Junam’, ‘P47JB-4-B-5-B’ had a similar grain appearance of brown rice as ‘Junam’, both of their grains seemed more round than those of ‘Hwayoung’. However, ‘P47JB-4-B-5-B’ had reduced 1000-grain weight compared with donor cultivar, ‘Hwayoung’ (Table 1). In this study, disruption of chromosome balance due to T-DNA insertion could explain why ‘P47JB-4-B-5-B’ showed a shorter plant height, grain weight, and yield than those of the donor and parental cultivars. Koh *et al.* (1993) previously reported an MNU-treated giant embryonic line with only 74.5% 1000-grain weight of that of ‘Hwacheong’. Maeda *et al.* (2001) reported that giant embryonic cultivars tend to have reduced 1000-grain weight. In addition, giant embryonic lines have a reduced ratio of ripened grains due to relatively bigger embryos (Koh *et al.*, 1993). In this study, the ratio of ripened grains of ‘P47JB-4-B-5-B’ was successfully improved via backcross with ‘Junam’.

Embryonic characteristics of ‘P47JB-4-B-5-B’

The 100-embryo weight of ‘P47JB-4-B-5-B’ was 0.134 g, which was 2 times heavier than that of ‘Hwayoung’. ‘P47JB-4-B-5-B’ showed a ratio of embryo to grain of 6.7%, which was 2.2 times that of ‘Hwayoung’ (3.1%). Its embryo length, width, embryo weight, and grain weight showed significant differences at a level of 0.01 compared to those of the donor cultivar ‘Hwayoung’ (Table 2, Fig. 1). Maeda *et al.* (2001) reported that the giant embryo cultivar ‘Haiminori’ has a 4 times heavier embryo weight than that of the donor cultivar ‘Nipponbare’, and the ratio of embryo to grain is 8.78%, but that of the common cultivar is only 1.88%. Zhang *et al.* (2005) reported a giant embryo cultivar, ‘XS-gel’, from a japonica rice cultivar ‘Xiushui 110’ treated with X-ray irradiation, with a 4.79% ratio of embryo to grain.

Chemical compositions of ‘P47JB-4-B-5-B’

The total free amino acid content of ‘P47JB-4-B-5-B’ was 75.81 mg, which was 2.1 times that of ‘Hwayoung’ (35.29 mg). Of the amino acids, the contents of serine,

Table 1. Comparison of brown rice between ‘P47JB-4-B-5-B’ and donor ‘Hwayoung’ or crossing parent ‘Junam’.

Lines and cultivars	Brown rice				
	Length (mm)	Width (mm)	Thickness (mm)	Ratio of length/width	1000-grain weight (g)
P47JB-4-B-5-B	4.84±0.14	2.92±0.09	2.11±0.04	1.66±0.04	19.91±0.27
Hwayoung	5.09±0.08	2.93±0.05	2.07±0.06	1.74±0.03	22.47±0.21
Junam	4.82±0.10	2.91±0.12	2.05±0.09	1.66±0.88	21.47±0.08

The data are presented in Mean±SD.

Table 2. Embryo size and weight of ‘P47JB-4-B-5-B’.

Line and cultivar	Embryo size (mm)		100-weight (g)		A/B(%)
	Length	Width	Embryo (A)	Grain (B)	
P47JB-4-B-5-B	3.00±0.19	1.47±0.13	0.134±0.002	1.991±0.030	6.7
Hwayoung	2.33±0.12	0.97±0.06	0.070±0.002	2.247±0.02	3.1
T-value	4.31**	5.19**	71.43**	-10.52**	

The data are presented as Mean±SD.
**Significant differences at 1% level from the donor cultivar

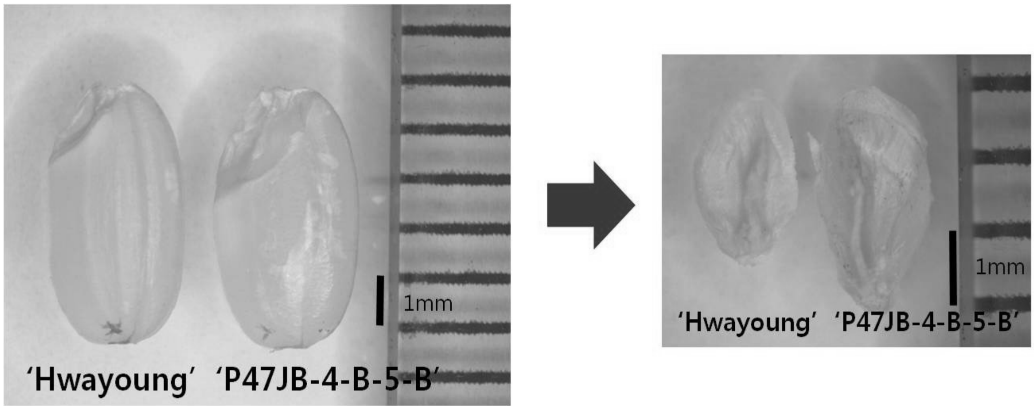

Fig. 1. Comparison of grain appearance and embryo size between 'P47JB-4-B-5-B' and the donor cultivar 'Hwayoung'.

Table 3. Free amino acids contents of the giant embryo line 'P47JB-4-B-5-B' and the donor cultivar 'Hwayoung' (mg/100g)

Amino acids	P47JB-4-B-5-B (A)	Hwayoung (B)	A/B (%)
Gamma-amino-n-butyric acid (GABA)	14.07	6.84	206
Threonine	1.82	0.79	230
Serine	4.45	1.59	280
Aspargine	8.83	6.59	134
Glutamic acid	6.79	3.35	203
Glycine	1.87	0.71	263
Alanine	7.21	3.53	204
Valine	2.70	1.20	225
Methionine	1.04	0.48	217
Isoleucine	0.98	0.42	233
Leucine	3.81	1.77	215
Tyrosine	2.08	0.90	231
Phenylalanine	2.82	1.39	203
Ethanolamin	0.85	0.55	155
Ammonium Chloride	1.10	0.87	126
Lysine	4.06	1.54	264
Histidine	1.11	0.51	218
Arginine	10.22	2.44	419
Total	75.81	35.49	214

glycine, and lysine, and especially GABA, were higher than those of the donor cultivar (14.07 mg/100 g). Furthermore, the contents of necessary amino acids, such as threonine, valine, and methionine, showed, were 2-2.6 times higher than those of the donor cultivar (Table 3).

The contents of eight amino acids in cultivar (Keunnun) with giant embryo were analyzed previously by Choi *et al.* (2009). The results indicate that the aspartic acid, serine, glutamic acid, glycine, cystine, GABA, and hydroxyl-lysine contents were higher than those of the donor cultivar 'Ilpum'. The total amino acid content was 97.60 mg/100 g as well as 1.8 times higher than that of donor cultivar 'Ilpum' (53.96 mg/100 g). Especially, the glutamic acid content was highest (30.58 mg/100 g). Saikusa *et al.* (1994) reported that the GABA and arginine can be assumed to contribute to the taste of cooked rice. GABA and arginine

Table 4. Changes in free sugar and GABA contents in germinated grain of the giant embryo line 'P47JB-4-B-5-B'.

Line and cultivar	Days after germination	Free sugars (%)				GABA (mg/100 g)
		Fructose	Glucose	Sucrose	Maltose	
P47JB-4-B-5-B	0	-	0.58	1.43	3.00	1.48
	1	0.41	1.07	0.53	3.04	18.70
	2	0.50	1.84	0.54	3.75	44.81
Hwayoung	0	-	0.50	0.90	3.30	1.16
	1	0.42	0.91	0.45	2.60	11.24
	2	0.68	1.88	0.40	3.13	17.43

were also analysed in 'P47JB-4-B-5-B' that is contained 32% of total amino acid.

After 24 hours and 48 hours of brown rice germination, the GABA and free sugar contents were determined. GABA contents of 'P47JB-4-B-5-B' were 33 times higher (18.70 mg and 44.81 mg, respectively) after germination compared to that before germination (1.48 mg), whereas GABA contents after 48 hours germination were only 6 times higher (17.43 mg) compared to that of before germination (1.16 mg). There were no significant differences in free sugar contents between 'P47JB-4-B-5-B' and 'Hwayoung' (Table 4). Significant increase in GABA content following brown rice germination has been reported by several researchers (Saikusa *et al.*, 1994; Zhang *et al.*, 2005; Choi *et al.*, 2006, 2009). Due to the hard taste, difficult cooking, and low digestion rate of brown rice, it is generally not favored by public consumers despite its higher nutritional content compared to milled rice. However, the outer layer of brown rice can be degraded by enzymes produced during the germination process, which makes the rice structure loose and induces the activity of many kinds of nutrients. In this case, cooking quality could be improved by increasing the water absorption factor (Choi *et al.*, 2004). Oh *et al.* (2010) reported that the content of GABA reached a maximum as the 10 mm of roots emergence during seed germination, whereas Choi *et al.* (2004) found that GABA reached its maximum content after soaking for 8 hours at 40℃. In this study, after pretreatment for 3 hours, the maximum GABA content of brown rice in 'P47JB-4-B-5-B' was observed after 48 hours of soaking at 30℃.

The free fatty acid contents of brown rice were analyzed for 'P47JB-4-B-5-B' and 'Hwayoung' as shown on Table 5. Palmitic acid, oleic acid, and linoleic acid accounted for 95% of the total fatty acid content. In addition, 78.9% and 79.4% of unsaturated fatty acids were observed in 'P47JB-4-B-5-B' and 'Hwayoung', respectively. On the contrary, the saturated fatty acid content of the former was relatively higher than that of latter. The same results were also reported by Fujino (1978) and Choi *et al.* (2009).

Table 5. Composition of free fatty acids in giant embryo line 'P47JB-4-B-5-B' and the donor cultivar 'Hwayoung'.

Fatty acid composition (%)	P47JB-4-B-5-B	Hwayoung
Myristic acid ($C_{14:0}$)	0.24	0.34
Palmitic acid ($C_{16:0}$)	16.50	16.55
Palmitoleic acid ($C_{16:1}$)	0.11	0.12
Stearic acid ($C_{18:0}$)	2.25	1.03
Oleic acid ($C_{18:1}$)	42.38	39.74
Linoleic acid ($C_{18:2}$)	36.55	39.63
Linolenic acid ($C_{18:3}$)	1.31	1.65
Arachidic acid ($C_{20:0}$)	0.40	0.52
Gadoleic acid ($C_{20:1}$)	0.26	0.42
Total	100	100

Genetic analysis for giant embryonic lines

The continuous frequency distributions of embryo length and width in 220 F_2 lines, which were derived from a cross between 'P47' and 'Cheongcheong', are shown on Fig. 2. Furthermore, high broad sense heritability of embryo length (86.6%) and width (86.7%) indicate that the giant embryonic lines could be selected in early generations (Table 6).

Previous studies have shown that one major gene or

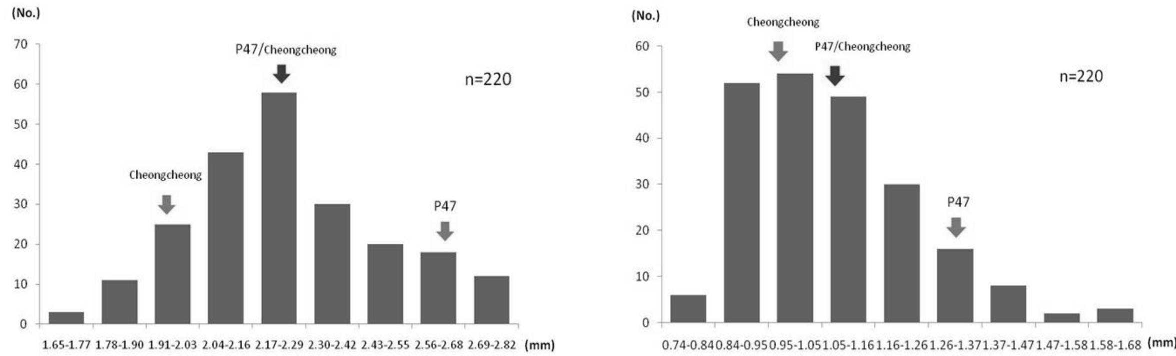

Fig. 2. Frequency distribution of embryo length and width in F_2 population from the cross between ‘P47’ and ‘Cheongcheong’.

Table 6. Heritability of embryo length and width in F_2 population from a cross ‘P47/Cheongcheong’.

Embryo size	Parents		T-value	F_2 population		
	P47 (P_1)	Cheongcheong (P_2)		Mean±SD	Range	H_2^b (%)
Embryo length	2.65±0.11	1.96±0.10	6.51**	2.25±0.24	1.65~2.82	86.60
Embryo width	1.29±0.09	0.95±0.06	4.63**	1.06±0.17	0.74~1.68	86.70

The data are presented in Mean±SD.
** indicates significant at 1% level
$H_2^b=(V_P-1/3(V_{P1}+V_{P2})/V_P*100\%$, it indicates the broad sense heritability of embryonic traits for F_2 population.

several QTLs can control embryonic traits (Koh *et al.*, 1996; Hong, 2002; Dong *et al.*, 2003; Lin *et al.*, 2009; Park *et al.*, 2009). Thus, the genetic map construction and genetic analysis for giant embryonic traits will be implemented in a future study.

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