Correlation Analysis between Grain Color and Cyanidin-3-glucoside Content of Rice Grain in Segregate Population

Tae-Ho Ham¹, Soon Wook Kwon², Su-Noh Ryu¹, Hee-Jong Koh^{3*}

¹Dept. of Agricultural Science, Korea National Open University, Seoul 110-791, Korea

²Dept. of Plant Bioscience, Pusan National University, Miryang 627-706, Korea

³Dept. of Plant Science, Seoul National University, Seoul 151-742, Korea

ABSTRACT This study examined the genetic variation of cyanidin 3-glucoside (C3G) contents in blackish-purple rice. F₂ populations were established from crosses between blackish purple rice and normal white rice. The blackish rice cultivars used were Jilinheimi, Heidao38, LK1A-2-12-1-1, Heugjinju, and No2, and the common white rice cultivars used were Hwachung super giant embryo and Heugbal. The purple pericarp color is known to be controlled by a set of dominant alleles, *Pb(Prp-b)* and *Pp(Prp-a)*. In this study, the segregation of the black purple:brown:white pericarp in the F₂ population was 9:3:4, demonstrating that the purple pericarp color was controlled by two dominant complementary genes. The F₂ distribution of C3G showed continuous variation, with a tendency toward a lower level in all the crosses. However, some F₂ crosses, such as Jilinheimi/Heidao38 and Jilinheimi/LK1A-2-12-1-1, showed transgressive segregation of the C3G content. C3G content of Jilinheimi linked on 84% level in C3G content of F₂ population between Jilinheimi/Heidao38, and 41.3% level in Jilinheimi/LK1A-2-12-1-1. The results of the color difference meter and C3G content analysed by HPLC revealed highly significant correlations between the seed coat color of the colored rice germplasm and that of the segregated populations. There was a significant positive correlation between the C3G content with *L** (lightness) and *b** (yellowness). The *a** (redness) was different in each cross, but there was a significant positive correlation between the C3G content with *L** and *b**.

Keywords Colored rice, Anthocyanin, C3G (cyanidin 3-glucoside), Color difference meter

INTRODUCTION

Annual rice consumption per person has dropped from 135.6 kg/capita/year in 1979 to 69.0 kg/capita/year in 2013. In conjunction with the drop in consumption, customers' requirements have diversified, with consumers demanding functional and healthful, as well as tasty rice. Various qualities of rice, such as its physical appearance, cooking properties, eating properties, and, more recently, nutritional value, affect consumers' acceptance of the product and the product's market value. Therefore, improving the rice varieties according to consumers needs were main objectives of rice breeding activities (Fitzgerald *et al.* 2009). The potential of colored rice as a functional food with health benefits (Choi *et al.* 1994; Kang *et al.* 1996), as well as its potential as a natural pigment (Choi *et al.* 1996)

and in cosmetics, has been studied.

In the past, the rice bran and germ produced during the milling process was disposed of, without utilizing these by-products of rice production. However, following research showing that rice bran includes a variety of useful components, such as oryzanol, phytic acid, ferulic acid, arabinoxylan, and arabinogalactan, its potential as a new functional ingredient has received increased attention. In particular, pigmented rice bran, rather than the bran of normal rice, is attracting attention because of its high antioxidant activity (Cho *et al.* 1996; Osawa 1995).

Genetic fortification of rice grain with functional compounds for human health is one of the major objectives in breeding programs for better and diversified grain quality in rice. Colored rice contains a variety of natural colors, ranging from dark purple to maroon and green (Park

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*Corresponding author Hee-Jong Koh, heejkoh@snu.ac.kr, Tel: +82-2-880-4541, Fax: +82-2-873-2056

et al. 1998). Black and dark purple rice are known to contain a large amount of anthocyanin pigments, whereas red and maroon rice contain tannins. The anthocyanin pigment of black-purple rice contains a large amount of cyanidin-3-glucoside (C3G), up to 80%, as well as peonidin-3-glucoside (P3G), malvidin, pelargonidin, and selphinidin (Reddy *et al.* 1994; Cho *et al.* 1996; Ryu *et al.* 1998).

C3G is the key component of anthocyanin, which is responsible for the color pigmentation in black-purple rice, and is known to have antioxidative potential (Reddy *et al.* 1995). A Korean blackish-purple rice cultivar, Heugjinju, was reported to contain 470 mg/100g of C3G, which is the highest concentration among cultivated rice in Korea (Kim *et al.* 2000). The grain color of black-purple rice is known to be controlled by the complementary action of two dominant genes, *Pb(Prp-b)* and *Pp(Prp-a)*, located on chromosome 4 and 1, respectively (Hsieh & Chang 1964; Yoshimura *et al.* 1997). The other grain color red also has complementary gene as *Rc* and *Rd* (Kinoshita 1984; Nagao & Takahashi 1956).

This study was carried out to investigate the inheritance of the concentration of C3G in segregate population and to explore the relationship between the intensity of pigmentation and the concentration of C3G.

MATERIALS AND METHODS

Plant materials

Five black-purple rice varieties, Jilinheimi, Heugjinju, Heidao38, No2, and LK1A-2-12-1-1, and two white rice varieties, Hwacheong super giant embryo and Heugbal, were used as parents of four populations. We performed two crosses each the black-purple variety/black-purple variety and black-purple variety/white variety. The black-purple/black-purple crosses were Jilinheimi/Heidao38 and Jilinheimi/LK1A-2-12-1-1, and the black-purple/white crosses were Heugjinju/Hwacheong-ges and No2/Heugbal.

The colored rices and white rices were crossed in a greenhouse experiment in the summer and subsequent generations were advanced in a greenhouse in the winter. The F_2 population was sown and transplanted in the central region of Korea. In the F_2 population, the plants were

harvested one by one, and F_3 seeds were selected from 150 plants. In the analyzed of the F_2 segregation ratio, unhulled seeds of F_3 were used, counted black-purple, red-brown and white seeds by visual, separately.

Phenotypic traits

Color measurements were expressed as tristimulus parameters (L^* , a^* , and b^*) with a color difference meter (Chroma meter CR-200, Minolta, Japan). L^* indicate lightness (100 = white, and 0 = black). a^* indicates redness-greenness (positive = red, and negative = green), and b^* indicates yellowness-blueness (positive = yellow, and negative = blue).

Extraction and detection of C3G

The C3G pigment from 2 g of brown rice was extracted and pulverized with 0.1% Trifluoroacetic acid (TFA) and 20 ml of 95% ethanol solvent, three times every 4 h (Kwon *et al.* 2011). The extracted solution was filtered with filter paper (Whatman No.2) and concentrated under reduced pressure. The extracted solution was filled up with 2 ml to HPLC analysis. C3G in anthocyanin extracted from the seed coat was analyzed with HPLC (waters 501 pump, millipore gradient controller, waters 480 UV-Vis detector). An ODS-5 column (4.6 mm×250 mm, Nomura Chemical Co., Japan) was used, and detect wavelength was 530 nm. The mobile phase was 0.1% TFA in water, the linear gradient was 0.1% in acetonitrile, and the flow rate was 1.0 ml/min

RESULTS

The segregation ratio of black:brown:white in the F_2 populations of crosses between the black and white rice was 9:3:4, in accordance with previous reports (Table 1). The segregation ratio differed in the F_2 population crosses between the black rice, there were all black. These results indicate that two dominant complementary genes control the color pigmentation in the black-purple pericarp and that the pericarps of Jilinheimi, Heidao38 and LK1A-2-12-1-1 possess the same genes for color pigmentation. However, the depth of pigmentation varied continuously within the

Table 1 Segregation of grain pericarp color in F₁ and F₂ of four crosses, Jilinheigmi/Heidao38, Jilinheimi/LK1A-2-12-1-1, Heugjinju/Hwacheong-ges, and No2/Heugbal.

Parents and crosses	Generation	Pericarp color of grain				χ^2 (9:3:4)	<i>p</i> -value
		Blackish purple	Dark brown	White	Total		
Jilinheimi	Parent	5			5		
Heidao38	"	5			5		
Heugjinju	"	5			5		
No2	"	5			5		
LK1A-2-12-1-1	"	5			5		
Hwacheong-ges	"			5	5		
Heugbal	"			5	5		
Jilinheimi/Heidao38	F ₁	All			4		
	F ₂	All			150		
Jilinheimi/LK1A-2-12-1-1	F ₁	All			-		
	F ₂	All			150		
Heugjinju/Hwacheong-ges	F ₁	All			3		
	F ₂	89	29	32	150	0.87	0.75-0.5
No2/Heugbal	F ₁	All			-		
	F ₂	87	34	39	150	2.14	0.5-0.25

Table 2. Correlation coefficients of evaluation methods of the seed coat color in F₂ seeds derived from a cross between Jilinheimi/Heidao38 and Jilinheimi/LK1A- 2-12-1-1.

Jilinheimi/Heidao38		X1	X2	X3	X4
Color	<i>L</i> * (X1)		0.442***	0.582***	-0.553***
Difference	<i>a</i> * (X2)			0.373***	-0.332***
Meter	<i>b</i> * (X3)				-0.653***
C3G (X4)					
Jilinheimi/LK1A-2-12-1-1		X1	X2	X3	X4
Color	<i>L</i> * (X1)		0.202**	0.756***	-0.697***
Difference	<i>a</i> * (X2)			0.139	-0.097
Meter	<i>b</i> * (X3)				-0.677***
C3G (X4)					

*L**: lightness, *a**: redness, *b**: yellowness **,***: significant at the 1% and 0.1% level, respectively

color classes, as measured by a color difference meter, implying that other genes might also be involved in the color expression (Fig 1). The C3G concentration of the brown rice exhibited continuous variations, with a tendency toward a lower value in all the F₂ populations (Fig. 2). C3G concentration varied widely, depending on the crosses and also found varying more or less transgressants. The variation in the C3G concentration in F₂ crosses did not

correspond with that of color pigmentation. However, there were significant correlations between the C3G concentration and *L** (lightness) and *b** (yellowness) values (Table 2).

Genetic segregation of grain color

Regardless of the combination, all the F₁ seeds were black, suggesting that a dominant gene affects the grain color. The F₂ seed grain color in the black/black combination,

Jilinheimi/Heidao38 and Jilinheimi/LK1A-2-12-1-1, was blackish, demonstrating that the parent of the black/black crosses had the same genes for black grain color.

The results of an χ^2 analysis of the segregation of the grain color in the F_2 population of the black/white crosses, Heugjinju/Hwacheong-ges and No2/Heugbal, was 9:3:4 (Table 1), indicating that Heugjinju and No2 has two genes associated with grain color and that these are complementary.

Segregation and heritability of C3G content

The F_1 and F_2 seeds of the black/black crosses were all black-purple, but the seed grain color of the F_3 seeds from the F_2 segregated generation showed a continuous variation, as shown in Figure 1. A colorimeter revealed that the shape of the C3G content in the F_2 population from a combination of the black/black crosses was similar. As expected, transgressive segregation occurred in the Jilinheimi/Heidao38 and Jilinheimi/LK1A-2-12-1-1 crosses (Fig. 2).

Fig. 1. Frequency distribution of color value a^* and b^* using a color difference meter in the F_2 population of Jilinheimi/Heidao38 (left) and Jilinheimi/LK1A-2-12-1-1 (right).

Fig. 2. Variation in the C3G content (mg/100g brown rice) of rice grain in F_2 of four crosses/populations. P indicates the C3G variation of the colored parents in the crosses.

Fig. 3. C3G concentration correlation between F_2 plants and derived F_3 lines. The standardized heritability estimate based on parent-offspring regression is the same as the correlation coefficient between generations.

Correlations between colorimeter score and C3G content

The C3G content showed a significant negative correlation with L^* (lightness) and b^* (yellowness), but different aspect showed with a^* (redness) by each combination (Table 2). In the Jilinheimi/Heidao38 combination, the lightness, redness, and yellowness had a highly significant negative correlation with the C3G content. The colorimeter values revealed a highly significant positive correlation with each L^* (lightness), a^* (redness), and b^* (yellowness) in these crosses. In the Jilinheimi/LK1A-2-12-1-1 combination, the C3G content had a significant negative correlation with L^* (lightness) and b^* (yellowness) but not with a^* (redness). The colorimeter values correlation in L^* (lightness) with b^* (yellowness) and with a^* (redness) were highly positive correlation, but negative correlation in a^* (redness) with b^* (yellowness). The correlation of the C3G content with L^* (lightness) and a^* (redness) differed, depending on the mating parents.

In particular, the separation aspects of the a^*/b^* was similar to the separation of the C3G content, which is thought to be due to a^*/b^* values representing the anthocyanin content (Fig 1 and Fig 2).

DISCUSSION

The potential of rice as a functional food has attracted attention in recent years, with research focusing, in particular, on dark purple rice. Since the early 1990s, dark purple crop varieties have been introduced from China.

Heugjinju (Moon *et al.* 1998) and Heugnam (Ha *et al.* 1998) has developed at rural development administration (RDA), but the yield of these varieties has remained at 70% as compared to that of normal varieties (Jung 2000).

The aim of the present study was to obtain basic data on color of pericarp using some pigmented rice varieties and to investigate the genetic underpinning selection for a high C3G content. The C3G content of some lines in the F_2 population was considerably higher or lower than that of the parents. This phenomenon is referred to as transgressive segregation. Transgressive segregation for quantitative traits is very common, but the genetic basis is largely unknown (Liang *et al.* 2009). Transgressive segregation is a quantitative trait, and the C3G content is controlled by a large number of genes. The Jilinheimi/Heidao38 and Jilinheimi/LK1A-2-12-1-1 combinations showed marked separation transcendence. The objects are separated beyond seems to be due to the cumulative effect or interaction effect of the gene to promote C3G content thought to be useful in the selective breeding. The action of complementary genes may be the primary cause of transgression, although overdominance and epistasis also contribute (Rieseberg *et al.* 1999). In the present study, standardized heritability of the C3G concentration estimated through selected F_2 and F_3 experiments was significantly high. These are suggest that selection for the C3G concentration in early generations would be effective.

Studies have suggested that the genetic systems conferring color pigmentation and the C3G content might not be the same but inter-related in a specific pathway

associated with anthocyanin metabolism. This study can be partly explained by the fact that significant variations exist in the concentration of C3G, even among black-purple rice varieties (Park *et al.* 1998; Ryu *et al.* 1998). The results of χ^2 analysis of the segregation of F₂ population's grain color in black/white crosses, Heugjinju/Hwacheong-ges and No2/Heugbal was 9 : 3 : 4. Jung *et al.* (2000) and Park *et al.* (2000) reported the same segregation ratio in Heugjinju/IR701078-AC3(white) crosses and Hyangmi (white)/Heugjinju crosses. Shen *et al.* (2009) reported that the color parameters of rice grain were positively correlated with the total phenolic content, flavonoid content, and antioxidant capacity among a wide collection of rice germplasms, including white, red and black rice, but that the correlations among the white rice accessions were rather weak. Jung *et al.* (2000) reported that the C3G content of an F₂ population had a highly significant negative correlation with colorimeter *a** (redness) with *b** (yellowness).

To shed light on the mechanism underlying transgression, it will be necessary to examine the interaction and activity of the dominant allele on heterozygote (Dooner *et al.* 1991). It will also be necessary to investigate the composition and quantitative differences in anthocyanin in the tissues of other plants, such as corn, snapdragon, and petunia, to shed light on the interaction between the structure and function of regulatory genes involved in the biosynthesis of anthocyanins.

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