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Genetic Analysis of Complementary Gene Interactions of *Pb* and *Pp* Genes for the *Purple Pericarp* Trait in Rice

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The *Purple pericarp* (*Prp*) trait is a trait often bred for in black rice. Generally, the *Prp* trait is displayed in the color variations of seeds following the 9:3:4 purple, brown, and white ratio, respectively. The *Prp* trait is a recessive epistasis of two gene interactions; however, it is caused by the two complementation genes *Pb* and *Pp*. Here we present a study of the genetic characteristics of the *Prp* trait using an F_1 hybrid with a *Pbpb Pppp* genotype. This hybrid generated four seed colors with the following numbers: 3 dark purple, 6 medium purple, 3 brown, and 4 white (or 9 purple, 3 brown, and 4 white). However, further biochemical analysis of the all progenies divided them into two groups. One group had the *Pb_ Pp_* allelic constitutions and contained cyanidin 3-*O*-glucoside (C3G) in both the dark purple or medium purple seeds. The other group, however, was absent of C3G in both the brown and white seeds, resulting in a ratio of 9:7, respectively. This segregation revealed the extended Mendelian 9:7 ratios of the complementary gene interactions with a good fitness in χ^2 analysis. Further analysis revealed that brown seeds with the *Pb_ pppp* genotype corresponded with a null C3G, indicating that the *Brown pericarp* trait in rice is caused by a dominant allele of the *Pb* gene. Therefore, we conclude that the production of C3G is a main phenotype of the black and purple colored rice in the *Prp* trait, and it is governed by the complementary gene interactions between *Pb* and *Pp* genes.

Key words : Black rice, complementary gene, epistasis, *Oryza sativa*, purple pericarp

Introduction

Rice (*Oryza sativa* L.) is the major cereal crop for consumption of the world's population and contains different colors such as white, red, brown, green and black [1, 4, 9-11]. Generally, the commercial name 'black rice' referred to the dark purple colored pericarp seed of rice, which is represented by genetic *Purple Pericarp* (*Prp*) trait (http://www.gramene.org) [2]. The black colored seeds are resulted by high accumulation of purple colored anthocyanin pigment in a single layer of cells in the pericarp of seed [10, 14, 15, 25]. The major anthocyanin pigments of black rice are cyanidin-3-*0*-glucoside (C3G) predominantly and peonidin-3-*0*-glucoside (P3G) additionally [5, 14, 16] . Because the C3G in cereals exhibited anti-cancer, anti-oxidant and anti-inflammatory activities, black rice was consumed widely as one of health promoting grains [3, 8, 18, 20]. Therefore, the rice *Prp* trait is a useful model in genetics of the purple colored pericarp cereals including wheat and barley [22, 23].

Two dominant genes, *Pb* and *Pp* [2, 14, 17, 21, 26], controlled the *Prp* trait in rice. Simply, it explains that the genetic constitutions in the ratio of 9 *Pb-Pp-* for purple : 3 *Pb-pppp* for brown : 4 *pbpbPp-* or *pbpbpppp* for white pericarps resulted in segregation of 9:3:4 ratios to support towards recessive epistasis [2]. However, the purple colored seeds of progenies were divided in the dark purple type and the medium purple type depending on parental genotypes gave ambiguous in the recessive epistasis [14], suggested that the phenotype of the *Prp* trait is not perfectly fit for the recessive epistasis in some genotypic classes by two gene interaction. Thus, genetic interactions of the genes in *Prp* trait are somewhat confusing in the recessive epistasis manner or the complementary gene interactions and need to be clarified further. After 97 years passed from genetic and physiological studies of black rice by Nagai [10], only the *Pb* gene of *Prp* trait has well defined DNA sequence in chromosome 4 and named *OsB1* that encodes a basic helix-loop-helix (bHLH)

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type transcription factor [14, 17, 21]. Therefore, further investigation is achievable to define the genetic definition of *Prp* trait for black rice.

Although high demand of black rice for healthy functional foods, the yield and eating quality of black rice are relatively inferior to that of white rice [12, 21, 26]. Many black rice cultivars have been breed with improved agronomical traits, but eating quality and yields still need to be improved [19, 23]. Recently, by using DNA markers covering the rice genome, Maeda et al. [6] reported that Japonica type black rice was developed with superior in eating quality to other black rice varieties, but showed slightly lower yields. Up to date, because of physiological factors and complicate genetic constitutions of two gene interactions, high yield black rice breeding is still hard than white rice breeding. One suggested that due to the high anthocyanin deposition in seed pericarp of black rice, chloroplasts in seed pericarp reduced the photosynthesis, resulting in yields reduction [13, 19].

Moreover, genetic complication of *Prp* trait is also a negative factor to black rice breeding. For example, allelic combinations of *Pb* and *Pp* are involved in resulting not only to produce various phenotypes of seed color appeared but also heterozygous progenies continued segregations [14]. Therefore, to breed black rice with enrichment in grain color, good taste and improved yield, the accurate predictions of genetic analysis of *Prp* trait are highly desired.

Taken together, we have some complications in analysis for the genetic constitutions of the progenies of black rice hybrids. The genetics and physiological natures of *Prp* trait were misinterpreted the segregation based on visible colors and it required further extended selection. In this study, we discuss the genetic definition of *Prp* trait of rice and suggests for the organization of the allelic patterns of *Pb* gene to yield increasing with enhanced C3G deposition in black rice.

Materials and Methods

Plant materials

We crossed a black gain rice cultivar 'Heugnambyeo' (YUC020) as a pollen recipient and white grain rice 'Ishehikari' (YUC044) as a pollen donor. Both are *Oryza sativa* L. Japonica type. The crosses were grown and selected from 2009 to 2017 in the rice paddy field at Yeungnam University, Gyeongsan, Korea.

Phenotype analysis and agronomic data scoring

The phenotypes of the purple pericarp plants and white pericarp plants were recorded [7]. In addition to the pericarp color of the materials, several agronomic traits including days to heading (DH), tiller number (TN), culm length (CL), leaf length (LL), plant height (PH), panicle exertion ability (PE), panicle length (PL), panicle number (PN), panicle thresh ability (PT), spikelet number (SN), spikelet fertility (SF) and 100-grain weight were evaluated. Specifically, plants in each line were evaluated for each type of agronomic data considered. The heading date for each plant was recorded as the first developing panicle to emerge approximately 1 cm beyond the leaf sheath of the flag leaf. For grain weight, 100 ripped spikelets were de-hulled and the weight in grams was measured.

Genetic analysis

The inheritance patterns of the pericarp color were analyzed by following previous study [14]. To evaluate the inheritance pattern of purple pericarps, segregation analysis of the purple pericarps was carried out using progenies of crosses. Segregation analysis and selection for pericarp color were performed to F_7 progeny. The genotype of the parents was determined based on the seed pericarp color phenotypes of the F_1, F_2 and progenies. Determination of dominant or recessive alleles of *Pb* genes among progenies was performed based on PCR-based polymorphism of the *OsB1* nucleotide sequences (accession number U39860) [14, 21]. Briefly, in the *OsB1* DNA sequences of a coding region of *Pb* gene, the recessive allele *pb* of the white rice showed the 2 bp (GT) insertion. In the GT deleted sequence of the dominant *Pb* allele offered a 5′-GGATCC sequence, which was cut by BamH1 restriction enzyme. However, the recessive *pb* allele offered a 5′-GGATGTCC sequence, uncut by BamH1 restriction enzyme. To accomplish the restriction enzyme assisted polymorphism of *Pb* genes, we used primers for *OsB1* sequences for a forward primer 5′-GGGAGAAGCTCAACG AGATG and a reverse primer 5′-GGGTGGCAGATTCATC ACTT. The 1.2 kb of PCR amplified fragments was purified and digested with *Bam*HI and then electrophoresed on 1.2% agarose gel to determine allelic constitution of each plant [14].

Cyanidin-3-O-glucoside detection

C3G was measured by following methods. One gram rice sample was taken and ground in to powder form. Methanol

extract was prepared by using a solution of 15 ml HCl/methanol (0.15% HCl/methanol) for 4 hr and this process was repeated three times. The extracts were then centrifuged at 10,000× *g* for 20 min and passed through a 0.25 μm PVDF filter (Millipore, Billerica, MA, USA). The filtered samples (10 μl) were subsequently injected into an HPLC (high performance liquid chromatography) (Sheseido, Tokyo, Japan) system. Separation was conducted using a CAP CELL PAK C18 column (4.6x250 mm; Sheseido) at 30℃ with the detection absorbance set at 520 nm. Kuromanin (Sigma, St. Louis, USA) was used as standard chemicals for measurement of C3G. C3G contents were determined as follows: C3G content = Concentration of standard C3G × (area of sample peak/area of C3G peak) × (diluted sample / weight of sample).

Results

Analysis of genetic constitution of *Prp* trait

The segregation pattern of pericarp colors in pedigree analysis of *Prp* trait was analyzed in Fig. 1. The cross named SGK09200 produced F_1 seeds with the medium purple pericarp seeds, demonstrated that black rice (YUC020) should have the homozygous dominant alleles of *PbPb PpPp* genes and white rice (YUC044) should have the homozygous recessive alleles of *pbpb pppp* genes. Therefore, we obtained a complete heterozygous F_1 plant with *Prp* trait consisted in alleles of the *Pb* and *Pp* genes. However, the F_2 generation segregated various colored pericarp seeds. Mainly, the segregation pattern of pericarp colors in the F_2 rice seeds clearly appeared novel phenotypes such as medium purple and brown purple compared with parental type as dark purple and white (Fig. 1A). The 259 offspring of the F_2 plants classified with 165 plants of purple pericarp seeds both dark and medium purple, 55 plants of brown pericarp seeds and 75 plants of white pericarp seeds. The segregation ratio showed 9 purple : 3 brown : 4 white (χ^2= 0.02, p<0.99) (Table 1). The Mendelian ratio 9:3:4 fit to recessive epistasis of two gene interaction. Indeed, the purple seeds consist of two types, dark purple and medium purple, depending on the degree of color intensity. Therefore, self-pollination of F_1 purple seed individuals showed four possible patterns outcomes with dark purple, medium purple, brown and white pericarp colors. This resulted that the ratio of the four color patterns was the ratio of 3 dark purple : 6 medium purple : 3 brown : 4 white (χ^2= 1.5, 0.90<p<0.50) (Table 2).

Genotype and phenotype analysis of the progenies

We further analyzed segregation patterns of color phenotypes of seed pericarps how correspondent to genotypes and chemical phenotypes of the selected progenies (Fig. 1B). Pigments were extracted from the seeds of black rice YUC020 and its progenies of the 7th generations including F_7-43 and F_7-32 plants. The reddish purple pigment was extracted from dark purple pericarp seeds but pink pigment was extracted in medium purple pericarp seeds of F_7-32.

Table 1. Segregation analysis in the cross between purple pericarp rice and white pericarp rice based on the recessive epistasis of the gene interaction

	F_2 segregation				χ2 (9:3:4) Df=2	*p*-value
Color phenotype	Purple	Brown	White	Total		
Ratio	(9)	(3)	(4)			
Genotype	*Pb_ Pp_*	*Pb_ pppp*	*pbpb Pb_, pbpb pppp*			
Observed	165	55	75	295		
Expected	165.94.	55.31	73.75	295	0.02	<0.99

Segregation ratio is 9:3:4.

Table 2. Segregation analysis in the cross between purple pericarp rice and white pericarp rice based on the inheritance pattern of seed pericarp color

	F_2 segregation					χ^2(3:6:3:4) Df=3	*p*-value
Color phenotype	Dark Purple	Medium Purple	Brown	White	Total		
Ratio	(3)	(6)	(3)	(4)			
Genotype	*PbPb PpPp*	*PbPb Pp_*	*Pb_ pppp*	*pbpb Pp_, pbpb pppp*			
Observed	40	125	55	75	295		
Expected	55.31	110.63	55.31	73.75	295	1.50	0.9~0.5

Segregation ratio is 3:6:3:4.

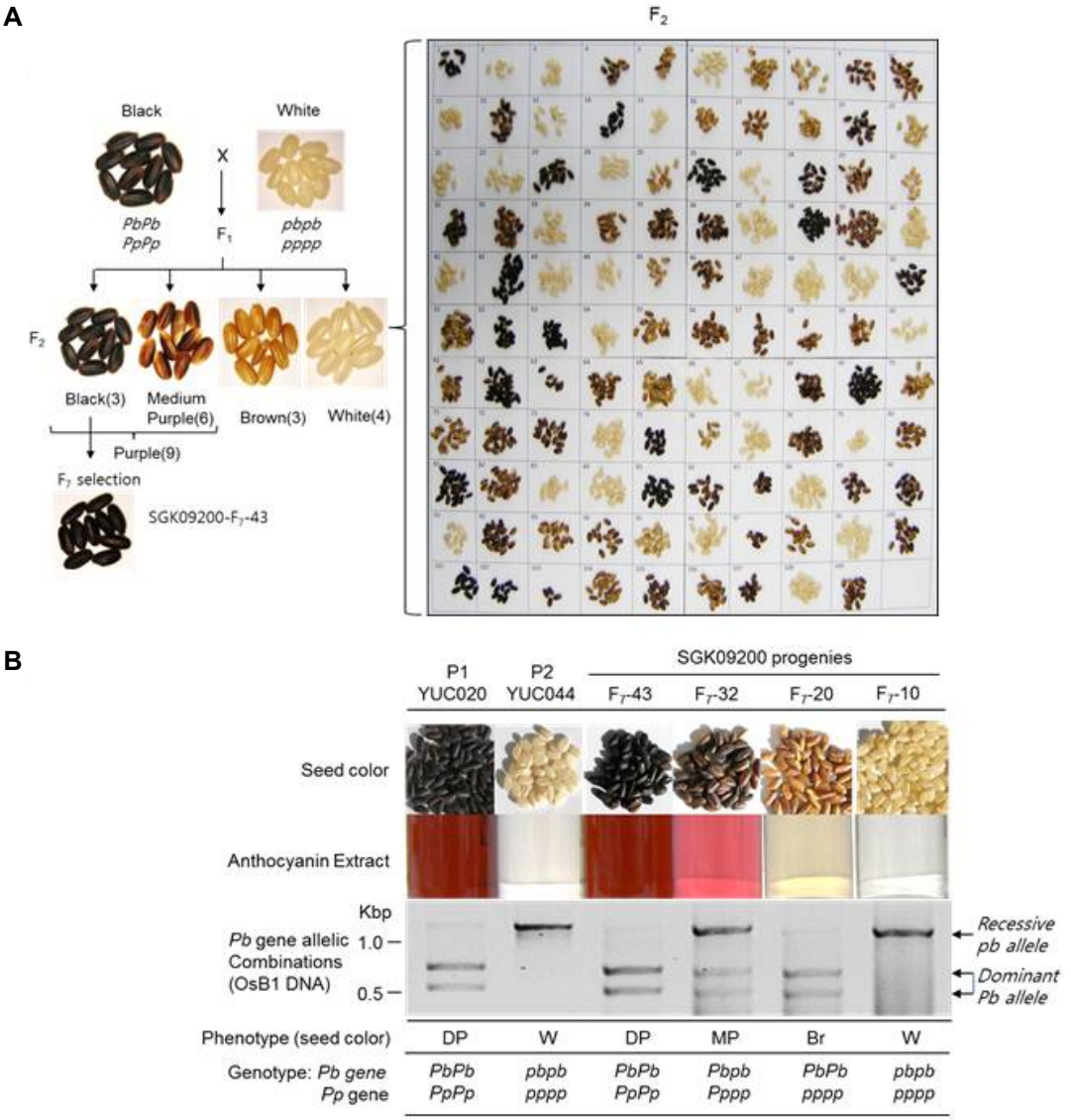

Fig. 1. Segregation pattern and allelic analysis of pericarp colors in rice seeds. (A) Black rice with dark purple pericarp (*PbPb PpPp*) was crossed with white pericarp rice (*pbpb pppp*) and named SGK09200. F_1 plants produced medium purple pericarp rice (*Pbpb Pppp*). F_2 plants produced four different color types of seeds; dark purple, medium purple, brown and white in pericarps were presented in the left panel. In the F_2 rice seeds, medium purple and brown were appeared as novel phenotypes, not in the parental types. SGK09200-F_7-43 was continued to be selected through seven generations. Black rice YUC020 'Heugnambyeo' used as pollen recipient. White pericarp rice YUC044 'Ishehikary' used as pollen donor. (B) Allelic polymorphism among progenies of the crosses between black pericarp rice and white pericarp rice. The pigments were extracted by 70% ethanol from each seeds. Red and pink colors retained by C3G. The rice allelic genotypes of *Pb* gene were determined using *Bam*H1 restriction enzyme digestion of *OsB1* DNA fragments. Recessive *Pb* allele (1.2 kb) was not digested with *Bam*H1 because of 2 bp (GT) insertion in the restriction site. Dominant *Pb* allele was digested with *Bam*H1 resulted in divided into two DNA fragments of 0.5 kb and 0.7 kb in the length. Phenotypes and genotypes are indicated by the pericarp colors, with 'DP' indicating dark purple, 'MP' indicating medium and mixed purple, 'Br' indicating brown and 'W' indicating white.

However, the extracts of brown pericarp seeds of F_7-20 plant showed light yellow (Fig. 1B). No pigment extracted from white pericarp seeds of YUC044 and F_7-10 (Fig. 1B). By liquid chromatography analysis, cyanidin-3-*O*-glucoside was contained only in the red and pink pigments of seed extracts in the Fig. 1B (Fig. 2).

To define phenotypic traits in seed color patterns and biochemical characters of the selected progenies were determine using *Pb* allelic polymorphisms. Previous researchers discovered that the *Pb* alleles are coded OsB1 protein, which

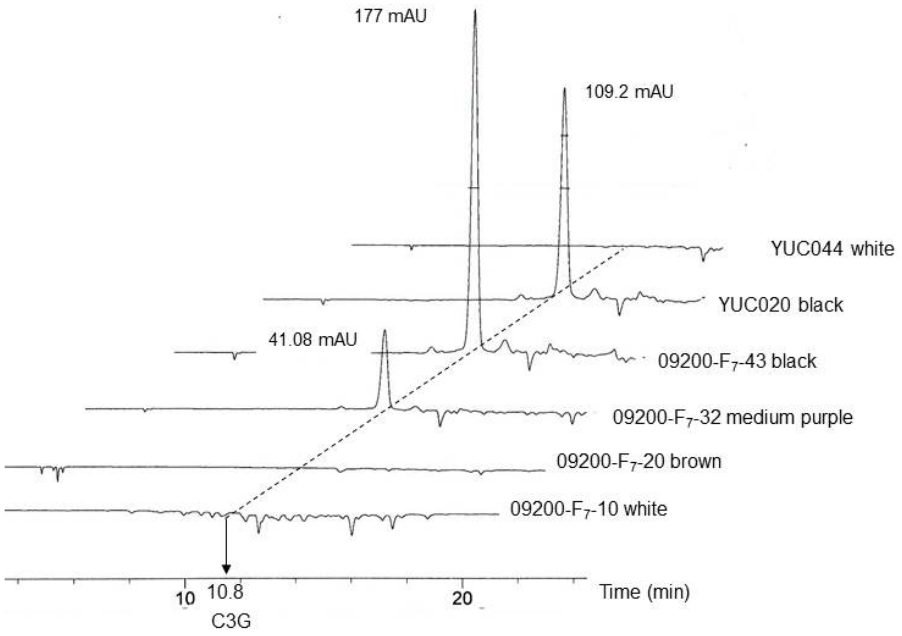

Fig. 2. Cyanidin-3-*O*-glucoside profile of black rice and white rice. HPLC profiles were obtained of extracts from the black (dark purple), medium purple, brown or white pericarps of rice seeds of the F_7 progenies. Parents were YUC020 and YUC044. The peaks in 10.8 minutes of retention time are C3G. A high amount of C3G was detected in the '09200-F_7-43' (*PbPb PpPp*). There were no detectable peak of C3G in the white seed (*pbpb pppp*) and brown seed *(PbPb pppp)*. Retention time is shown on the horizontal line and the amount of absorption unit (mAU) is shown on the each peak.

functions as a basic helix-loop-helix transcription factor [17, 21]. Therefore, allelic genotypes of *Pb* gene in the progenies were determined by PCR based polymorphisms using a specific primer set presented in the part of materials. In the *OsB1* DNA sequences, a *Bam*H1 restriction enzyme site is presented in the dominant allele, but absented in a recessive *pb* allele. Therefore, *Bam*H1 restriction digestion of the 1.2 kb PCR products of *OsB1* DNA from purple rice produced two DNA fragments, while those of white pericarp rice were not restricted [8]. As shown in figure 1B, further polymorphism analyses were revealed that the gene constitutions of homozygous *PbPb* dominant alleles were two dark purple (DP, black) include YUC020 and F_7-43 and a brown (Br) F_7-20, indicating that *Pb* gene is not directly determine to purple color deposition by C3G synthesis. Meanwhile, white seeds phenotype of YUC044 and F_7-10 were revealed homozygous recessive alleles of *pbpb* gene constitution. However, the medium purple pericarp (MP) of F_7-32 showed three bands, indicating that MP phenotype was consisted with heterozygous alleles of *Pbpb* gene constitution. To make an additional remark, the alleles of *Pp* gene were determined by segregation analysis in generations of progenies (Fig. 1B). Pigment production was required at least one dominant allele in the two genes *Pb* and *Pp*. However, the degree of color intensity in the seed extracts was appeared to be determined by the numbers of dominant alleles of the two genes.

Contents of cyanidin-3-O-glucoside in genotypes

Previously, C3G was determined by the number of dominant *Pp* alleles [14]. Consistently, we confirmed yet again to support the function of *Pp* gene by measuring the quantity of C3G of the experimental plants (Fig. 2). As shown in the HPLC data, relatively high amount of C3G was contained in the dark purple phenotypes of YUC020 and F_7-43 (Fig. 2, Table 3). The F_7-43 contained about 316.8 mg/kg of C3G. However, no C3G peak identified in the white pericarp seeds of YUC020, F_7-10 and the brown pericarp seed of F_7-20 (*PbPb pppp*). Here, we clearly identified that brown pericarp F_7-20 of *PbPb pppp* genetic constitution did not produce C3G (Table 3). The number of copies of dominant *Pb* and *Pp* alleles determined the level of C3G. Additionally, biochemical phenotypes of the progenies demonstrated that only two phenotypes are presented either C3G contained or absent (Table 3, Fig. 1B). Therefore, we rearranged new segregation ratio instead of the ratio 9:3:4(Table 1). When we consider the biochemical phenotype of cyanidin-3-*O*-glucoside content, it gives rise to a good fit to a ratio of 9 present C3G

Table 3. Quantification of cyanidin-3-*O*-glucoside in the progenies for definition of biochemical phenotypes compared with visible color phenotypes and genotypes

Plant	Phenotype	Genotype	Con. of standrd (mg/kg)	Area of standard	Area of sample	Weight of sample used (g)	injected sample (ml)	Weight of C3G (mg/kg)	Weight of C3G (mg/g)
SGK09200-F_7-10	white	*pbpb pppp*	50	9110	0	1	20	0	0.000
SGK09200-F_7-20	brown	*PbPb pppp*	50	9110	0	1	20	0	0.000
SGK09200-F_7-32	medium purple	*PbPb Pppp*	50	9110	669.2	1	20	73.458	0.073
SGK09200- F_7-43	dark purple	*PbPb PpPp*	50	9110	2,886	1	20	316.795	0.317
YUC020	dark purple	*PbPb PpPp*	50	9110	1,799	1	20	197.475	0.197
YUC044	white	*pbpb pppp*	50	9110	0	1	20	0	0.000

**All measuring were performed three times repeated. C3G is cyanidin-3-O-glucoside.* s

Table 4. Segregation analysis of biochemical phenotype in the cross between purple pericarp rice and white pericarp rice based on the complementary gene action

	F_2 segregation			$\chi 2$ (9:7) Df=1.	*p*-value
Color phenotype	Purple	Brown or White	Total		
C3G Chemical phenotype	C3G exist	null			
Ratio	(9)	(7)			
Genotype	*Pb_ Pp_*	*Pb_ pppp, pbpb Pp_, pbpb pppp*			
Observed	165	130	295		
Expected	165.94	129.06	295	0.00	1

Segregation ratio is 9:7.

**C3G is cyanidin-3-O-glucoside*

: 7 absent C3G that displayed the complementary gene action between *Pb* and *Pp* genes (χ^2= 0, *P*=1) (Table 4).

Analysis of *Pb* allelic polymorphism for selection

To improve yields and C3G contents of black rice, we performed selections for progenies of the cross, 'Heugnambyeo' (YUC020) and 'Ishehikari' (YUC044), by *Pb* allelic determination by polymorphism analysis (Fig. 1B). Since the F_2 as well as next progenies were segregated continually in various different types with anthocyanin colors as shown in figure 1, it was hard to select the good breeding lines with high yield and improved quantity of anthocyanin. To overcome this, we selected some candidate progenies of homozygous *PbPb* allelic combination assisted with *Pb* genotyping (Fig. 1B). Finally, we selected the SGK09200-F_7-43 individual (Fig 3, Table 5). Specifically, the plant height of F_7-43 was 119.4 cm that taller than parental types and more tiller numbers than their parents (Table 4). Moreover, we find im-

Table 5. Comparative agronomic traits among purple and white pericarp rice

Cultivar	Pericarp color	Plant height (cm)	Culm length (cm)	Flag leaf length (cm)	Flag leaf width (cm)	No. of tillers per plant	No. of panicle per plant	Panicle length (cm)	No. of grains per panicle	Seed fertility (%)	100 seed weight (g)
YUC044 (Isehikari)	White	107.7±1.5	68.9±1.1	38.8±0.7	1.22±.03	16.2±1.4	14.6±1.4	18.0±0.2	111.0±4.9	98.2±1.1	2.25±.14
YUC020 (Heugnam-byeo)	Black	97.0±0.7	66.8±1.0	30.4±0.8	1.21±.02	15.3±1.4	14.0±1.3	16.1±0.3	120.4±5.5	98.9±1.1	2.04±.09
SGK09200-F_7-43	Black	119.4±0.8	78.9±1.1	39.0±1.8	1.34±0.07	20.9±1.8	25.3±1.0	22.0±0.5	160.1±1.2	98.63±2.0	2.50±.24

Data are means±standard error of five observations for each trait. SGK09200 crossed between 'Ishikari' (*O. sativa* Japonica) and 'Heugnambyeo' (*O. sativa* Japonica).

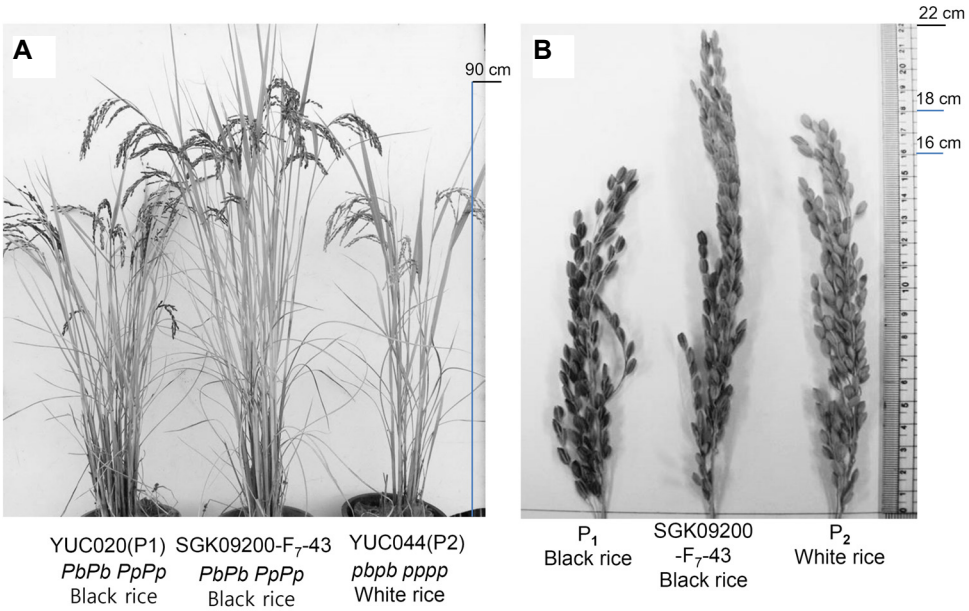

Fig. 3. Selected black rice progeny having homozygous *Pb* and *Pp* alleles. Plant features (A) and spikelets (B) were compared with pollen donor YUC044 (P1), pollen recipient YUC020 (P2) and a selected black rice line, SGK09200-F_7-43.

proved in tiller and panicle number per plant, panicle length, number of grains per panicle and 100 seed weight than those of parents (Table 5). The selected F_7-43 plant showed dark purple pericarp with 0.317 mg/g of cyanidin-3-*O*-glucoside that is relatively high percentage among parents and other progenies. We finally established that the genotype of the selected F_7-43 plant line showed the alleles of two genes constituted with homozygous pairs as *PbPb PpPp* (Fig. 1B, Table 3).

Discussion

Rice *Purple pericarp (Prp)* trait caused by the complementary gene action of *Pb* and *Pp* genes

Historically, rice *Purple pericarp* (*Prp*) trait has been defined the formation of purple color in seed pericarp named 'Black rice' [2, 10]. Further genetics determined that *Prp* trait is determined with recessive epistasis by the interaction of two genes, *Pb* and *Pp* [2, 17, 21]. The *Pb* gene linked in chromosome 4 and coded *OsB1* DNA sequence that encodes a helix-loop-helix transcription factor to control genes involved in the biochemical pathway to C3G synthesis [2, 17]. Basically, it explains that the genetic constituted ratio of 9 *Pb_ Pp_* purple : 3 *Pb_ pppp* brown : 4 *pbpb Pp_* or *pbpb pppp* non-color pericarps resulted in segregation to fit the Mendelian 9:3:4 ratios, supporting the recessive epistasis in genetic hypothesis [2]. However, the purple pericarp seeds of the cross between a black seed of homozygous dominant and a white seed of homozygous recessive divided two different patterns of seeds, such as dark purple and medium purple produced by alleles of *Pp* gene [14]. Briefly, the segregation pattern of the *Pp* alleles was correlated with the amount of anthocyanin in the segregation of 1 dark purple (*Pb_ PpPp*) : 2 medium purple (*Pb_ Pppp*). Hence, we obtained all F_1 showed medium purple, indicating that the F_1 plant consisted with heterozygous alleles of *Pbpb Pppp* genes (Fig. 1A). Based on our visible color variation of seed pericarps, the F_1 hybrid produced 4 different seed pericarp color types in F_2 progeny with the ratio of 3 dark purple : 6 medium purple : 3 brown : 4 white pericarp colored rice (χ^2= 1.5, *P*=0.90-0.50), suggesting that it is acceptable ratio of segregation (Table 2). However, the χ^2= 1.5 value of the analysis based on the ratio 3:6:3:4 is larger than the χ^2= 0.05 value of the ratio 9:3:4. This can be further clarified that some medium purple seeds to be considered as dark purple seeds due to undistinguishable visibility (Fig. 1A). Consequently, when 3 dark purple and 6 medium purple considered as one purple group, resulted in the ratio 9:3(br):4. However, the ratio 3:6:3:4 in phenotype segregation of the rice *Prp* trait was also acceptable in color visibility. Furthermore, the rice *Prp* trait in color visibility was indefinite with ambiguous for the recessive epistasis manner with the ratio 9:3:4 (Table 1). Certainly, rice is a true-breeding species, therefore, the non-parental phenotype of the medium purple (MP) should be true

originated by genetic combinations not to by physiological events. In here, when we accounted the segregated numbers of progenies depending upon visibility of seed colors, we also challenged to define the genetic interactions of the genes involved in the *Prp*. Up to date, because the genetic analysis based on seed colors is ambiguous, it has been understandable why the genetic interaction of this rice *Prp* trait was considered either the recessive epistasis (9:3:4) or the complementation interactions (9:7) of two genes, *Pb* and *Pp* [2, 14].

In fact, biochemical phenotype should be reflecting in a metabolic pathway mediated by the functions of proteins. Our further biochemical analysis clearly revealed that both dark purple and medium purple seeds had only C3G (Fig. 1B, Fig. 2 and Table 3). Analysis of biochemical phenotype showed a good fit to the ratio 9 presence C3G : 7 absence C3G in the F_2 generations (Table 4). In the analysis, the brown or white pericarp seeds does not contain C3G (Table 3) and consistently showed the same results of previous data [14]. Yet again, self-pollination of the F_1 hybrid generation generated F_2 progeny in the ratio of 9 presences C3G : 7 absence C3G. The extended Mendelian 9:7 ratios among the F_2 progeny established is true, supporting that a complementary gene interaction between *Pb* and *Pp* genes determine the black rice *Prp* trait. In conclusion, based on the true complementary gene interactions of rice *Prp* trait, the dominant alleles of two genes involve together (*Pb_ Pp_)* in the one metabolic pathway to produce C3G in the pericarp of rice. The other three genotypic classes, including *Pb_ pppp, pbpb Pp_* and *pbpb pppp*, could not synthesize C3G due to non-functional factors produced by recessive alleles of *pb* and *pp* to accomplish the pathway for C3G production, resulting in no purple colored pericarp seeds. Therefore, based on color visibility in previous reports, black or purple rice was caused by recessive epistasis gene interaction. But, we define here that *Prp* trait was presented by biochemical phenotypes which was resulted by complementary action by two different genes, *Pb* and *Pp*. This genetic definition of rice *Prp* trait may be applied basic genetics of the purple colored pericarp cereals, including wheat and barley [22, 24].

Analysis of *Pb* allele helps to black rice breeding

Rice *Prp* trait has been known as a manner of recessive epistasis (9:3:4) by previous genetic analysis based on visual phenotype [10]. This recessive epistasic trait made complicate and hardship for black rice breeding [14]. When we considered the ratio 9 of the segregation by the recessive epistasis of *Prp* trait, the purple pericarp progenies in cross between black and white rice could be considered 56.25% of purple pericarp progenies among all segregated progenies. Thus, it was expected that selection of the dark black rice with good agronomic characters in the population with high probability. However, when we selected the medium purple with visible color phenotype, the selected progenies were continued segregation in further generations. This caused by heterozygous *Pb- Pp-* genotypes and resulted in not only complicated but also required hard work in breeding for dark purple pericarp rice until achievement progenies having homozygous dominant pairs of *Pb and Pp* genes (Fig. 2 and Table 4). Indeed, because dominant *Pp* allele was incomplete dominance to recessive *pp* allele [14], homozygous *PbPb* allelic constitution was 1/3 of both and medium purple pericarp progenies. Therefore, only 18.75% among all segregated progenies of *Pb* and *Pp* genes was dark purple pericarp plants because of the ratio of 3 dark purple : 6 medium purple : 3 brown : 4 white (Fig. 1A). Furthermore, in the selected 18.75% progenies, the chance to select for plants with good agronomic characters was rare. In this simple assumption, the selection for dark purple black rice with good agronomic characters will be required to maintenance large population and repeat segregation analysis for years. This may be one of the disadvantages to breed a good black rice cultivar [12, 13]. However, as presented in the figure 1B, we performed genotypic determination of *Pb* allele in the group of purple pericarp progenies because plants with homozygous dominant *PpPp* and *Pb_* showed purple. Purely dark purple consisted with homozygous dominant alleles of *Pp* gene. However, one out of selected three dark purple progenies might consist with heterozygous alleles of *Pb* gene such as a *Pbpb PpPp* genotype, resulting in continuing segregation into dark purple and medium purple rice. Therefore, allelic determination to homozygous *Pb* alleles in purple progenies is very useful to select the dark purple line progenies. Moreover, when we determined *Pb* genotype of the selected dark purple lines, selection was more accessible to improve agronomic characters of black rice. By *Pb* gene alleles assisted genotyping, we precisely worked to improve high yield and agronomic characters of black rice with high content of C3G.

As we discussed in genetic constitutions of rice *Prp* trait, we selected F_7-43 plant that showed the alleles of two genes constituted as homozygous pairs as *PbPb PpPp* (Fig. 1B).

Furthermore, C3G concentration in the progeny SGK09200 F_7-43 increased 1.6 folds than the black parent YUC020 and 4.3 folds increased than medium purple (Fig. 2, Table 3). The selected black rice showed improved agronomic characters in tiller numbers, panicle length, grains in panicle and seed weight compared than those of parents (Table 5). Therefore, we demonstrated that black rice cultivar having better agronomic characters with increasing C3G could be selected by allelic analysis of *Pb* gene.

In conclusion, here we confirm that the rice *Purple pericarp* (*Prp*) is caused by the complementary action of two different genes, *Pb* and *Pp,* based on analyses of color phenotype, biochemical phenotype and DNA based genotype. In addition, the genotyping of *Pb* alleles would be useful in breeding black rice by reducing the efforts to select the good black rice lines in a massive progenies having seed color variations.

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초록 : 흑미의 자색종자과피(*Purple pericarp*) 형질을 결정하는 상보적 유전자 *Pb*와 *Pp* 유전자들의 상호관계 분석

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벼 자색 종자과피(*Purple pericarp, Prp*) 형질은 주요 생리활성물질인 안토시아닌 C3G 생성에 관여하며 흑미를 결정하는 주요 유전형질이다. *Prp* 유전형질을 가진 흑미와 종자과피에 색이 없는 벼를 교배할 경우 그 후대는 검정색, 갈색, 백색이 각각 9:3:4로 분리된다. 1921년 Nagai에 의하여 제시된 바 벼 종자 색의 9:3:4 유전분리비로 인하여 벼 *Prp* 형질은 유전자의 열성상위(recessive epistasis) 현상으로 해석되었다. 그러나 흑미를 결정하는 *Prp* 형질은 두 개의 상보적 유전자들의 상호관계(complementary gene interaction)에 의한 것이기도 하다. 본 연구에서는 이러한 논란이 발생되는 이유를 설명하기 위하여 두 유전자의 조성이 완전한 이형접합인 *Pbpb Pppp* 유전자형을 가진 F_1 잡종을 만들었다. 이들의 자손은 진한자색(검정), 중간자색, 갈색, 백색 종자이며 각각 3:6:3:4로 분리되었다. 즉, 검정색, 갈색, 백색의 종자가 각각 9:3:4의 비율로 분리된다. 그러나 생화학적인 분석결과 이들은 안토시아닌 중 cyanidin 3-O-glucoside (C3G)가 축적된 검정색 종자와 C3G가 없는 갈색 또는 백색 종자인 두 개의 집단을 분리되며 정확히 9:7의 분리 비를 갖는다. 이 경우 벼 *Prp* 형질을 갖는 검정쌀 또는 흑미는 전형적인 상보적 유전자의 상호관계에 의한 유전현상이다. 즉, 흑미의 자색 종피 형질 발현에는 *Pb* 유전자와 *Pp* 유전자에서 각각 한 개 이상의 우성대립인자의 발현이 필요하다. 그러나 *Pb* 유전자만 우성대립인자가 존재하는 *Pb_ pppp* 유전자형의 벼는 C3G를 생성하지 못하고 갈색 종자과피(*Brown pericarp, Brp*) 형질을 갖게 된다. 즉 갈색쌀는 우성 *Pb* 유전자의 우성대립인자에 의하여 결정된다. 그러므로 종피색을 결정하는 *Prp* 형질의 유전양상은 열성상위 현상으로 보이나 흑미의 결정요소인 안토시아닌 C3G의 함유 여부에 관한 유전분석을 시행하면 9:7의 비율로서 전형적인 두 개의 유전자가 모두 관여하는 상보적 유전현상이다. 유전적 정의는 유전자의 최종산물에 의한 물리적 또는 화학적 결정이다. 그러므로 결론하여 검정 쌀의 주요 생리활성물질인 안토시아닌 C3G 생성에 관한 유전현상은 *Pb*와 *Pp* 유전자의 상보적 유전자의 상호에 의한 것이다.