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ORIGINAL ARTICLE

The Genetic Constitutions of Complementary Genes *Pp* and *Pb* Determine the Purple Color Variation in Pericarps with Cyanidin-3-*O*-glucoside Depositions in Black Rice

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Abstract The purple pericarp color in rice was controlled by two dominant complementary genes, *Pb* and *Pp*. Crossing black rice 'Heugnambyeo' variants with three varieties of white pericarp rice gave a segregation ratio of 9 purple: 3 brown: 4 white. The *Pp* genes were segregated by homozygous *PpPp* alleles for the dark purple pericarps, heterozygous *Pppp* alleles for the medium and mixed purple pericarps, and homozygous *pppp* alleles for either brown or white pericarps with a 1 *PpPp*: 2 *Pppp*: 1 *pppp* segregation ratio, indicating that the *Pp* allele in rice is incompletely dominant to the recessive *pp* allele. Among the purple seeds, the amount of cyanidin-3-*O*-glucoside was higher in the dark purple seeds (*Pb_PpPp*) than in the medium purple seeds (*Pb_Pppp*). Moreover, no cyanidin-3-glucoside was detected in brown (*Pb_pppp*) or white pericarp seeds (*pbpbpppp*). These findings indicated that the level of cyanidin-3-glucoside was determined by the copy number of the *Pp* allele. Further genotype investigation of the F_3 progeny demonstrated that the dominant *Pb* allele was present in either purple or brown pericarp. A 2-bp (GT) deletion from the DNA sequences of the dominant and functional *Pb* was found in the same DNA sequences of the recessive and non-functional *pb* allele. These findings suggested that the presence of at least a dominant *Pb* allele was an essential factor for color development in rice pericarps. In conclusion, the *Pp* allele in rice is incompletely dominant to the recessive *pp* allele; thus, the number of dominant *Pp* alleles determines the concentration of cyanidin-3-*O*-glucoside in black rice.

Key words: Anthocyanin, Black rice, Epistatic interaction, *Prp*, *PURPLE PERICARP*

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Introduction

Rice cultivars have a variety of seed pericarp colors owing to black, brown, green, and red pigment deposition (Furukawa et al. 2006; Kang et al. 2006; Reddy et al. 1995; Sweeney et al. 2006). Among the various colors of rice, black rice is characterized by dark purple pericarps in seeds with high levels of anthocyanins. During rice seed development, purple pigments of anthocyanin accumulate rapidly in the pericarp, resulting in the characteristic dark purple grains of black rice (Abdel-Aal et al. 2006; Reddy et al. 1995; Shao et al. 2011). Previous genetic investigations have shown that cyanidin-3-*O*-glucoside and peonidin-3-*O*-glucoside are the two primary anthocyanin pigments deposited in the seed pericarps of black rice (Abdel-Aal et al. 2006; Hu et al. 2003; Jang and Xu 2009; Kim et al. 2007; Kim et al. 2011; Zhu et al. 2010). The pericarp pigmentation of black rice requires two genes, *PURPLE PERICARP A* (*Pp, Prpa* and *Prp1*) and *PURPLE PERICARP B* (*Pb, Prpb* and *Prp2*) located on chromosomes 1 and 4, respectively (Hu et al. 1996; Oryzabase, www.gramene.org; Wang and Shu 2007; Wang et al. 2009; Yoshimura et al. 1997). The *Pp* gene acts in a complementary fashion with the *Pb* gene for the production of purple[ED highlight – please ensure this is correct.] pericarps in rice (Hsieh and Chang 1964; Wang and Shu 2007). However, the

intensity of pericarp color in black rice varieties varies from dark purple to light purple, and the genetic mechanisms for these variations have yet to be identified.

Black rice is of interest owing to nutritional reasons. Indeed, many studies have demonstrated that consumption of the extracts of black rice bran reduced oxidative stress and increased antioxidant capacity in the animal models (Chung and Shin 2007; Ling et al. 2001; Min et al. 2011; Nam et al. 2006; Sompong et al. 2011). However, to breed black rice with good grain color, quality and high yields, accurate predictions of the genotypes of black rice are necessary. In this study, the various intensities of purple color in the progeny of black rice were shown to be inherited and controlled by complementary genetic action of the alleles of the *Pb* and *Pp* genes, resulting in additional problems associated with breeding of colored rice. Therefore, we investigated the genetic relationships between the genetic constitutions of the alleles of *Pb* and *Pp* genes and the inheritance patterns of black rice cultivars.

Results

Phenotypic Analysis of Purple Pericarp Rice

The rice germplasms *Oryza sativa* L. *japonica* var. 'Kewha' and *O. sativa* L. *japonica* var. 'Heugnambyeo' contained purple pericarps and seed coats during the mature stage. The color of the endosperm was white, as observed in wild-type rice. Several important agronomic traits were noted from the colored rice and white pericarp rice with respect to days to heading, plant stature, tillering ability, leaf structures, and panicle structure (Table 1).

The *Pb* and *Pp* Genes were Complemented for Pericarp Color Development

The pericarp color of all F_2 seeds from the F_1 plants generated by all cross-combinations displayed a purple phenotype, reflecting the dominant nature of the purple pericarp phenotype. Among 109 F_2 plants obtained from a cross between the purple pericarp rice 'Heugnambyeo' and white pericarp rice 'Hwayongbyeo', the seed pericarps of 56, 20 and 33 plants were purple, brown, and white, respectively (Table 2). We used the Chi-square (χ^2) test to evaluate the goodness of fit of the numbers of genes segregating in the population. The *Pp* and *Pb* genes, which are located on rice chromosomes 1 and 4, respectively, were necessary for pericarp pigmentation with the anthocyanins of black rice (Hu et al. 1996). Therefore, we scored the combination of these genes in the phenotypes of the progeny of the cross. Rice with the *pbpbPpPp* or *pbpbpppp* genotype produced white pericarp grains. Thus, we hypothesized that the *Pb* and *Pp* genes segregated according to the recessive epistatis ratio of 9:3:4. The results of χ^2 analysis of the F_2 segregation data fit with a ratio of 9:3:4 (purple: brown: white) (χ^2= 1.682, p-

Table 1. Several important agronomic traits of the studied white and purple rice germplasms

Plant ID	Pericarp	DH	CT	CL	PH	LL	LW	TN	PE	PL	PN	PT	SN	SF	GW
Hwayongbyeo	White	74±3	3	67.4±1.3	100.4±0.5	34.6±1.3	1.53±.02	8.60±0.7	1	23.3±0.6	7.5±0.7	3	141.2±7.7	88.6±1.7	2.74±.17
Kewha	Purple	71±4	3	62.4±0.8	89.7±0.5	27.3±0.9	1.05±.02	13.5±1.4	1	20.8±0.4	11.2±1.0	3	114.4±2.5	90.1±1.3	2.50±.16
Heugnambyeo	Purple	73±3	3	66.8±1.0	97.0±0.7	30.4±0.8	1.21±.02	15.3±1.4	1	20.1±0.3	14.0±1.3	3	120.4±5.5	91.9±1.1	2.44±.09

Note: DH; days to heading (defined as duration from transplantation to emergence of the first panicle), CT; culm thickness (Scale: 1; thin, 3; medium, 5; thick), CL; culm length in centimeter (cm), PH; plant height in cm, LL; leaf length in cm, LW; leaf width in cm, TN; number of reproductive tiller per hill, PE; panicle exsertion ability (Scale: 1; well exserted, 3; moderately well exserted, 5; just exserted, 7; partly exserted, 9; enclosed), PL; panicle length in cm, PN; panicle number per plant, PT; panicle thresh ability [Firmly grasp and pull the hand over the panicle and estimate the percentage of shattered grains. Scale: 1; difficult (less than 1%), 3; moderately difficult (1-5%), 5; intermediate (6-15%), 7; loose (26-50%), 9; easy (51-100%)]. SN; spikelet number per panicle, SF; spikelet fertility percentage, GW; 100 grains weight in gram, ±; standard error of five observation for each trait.

Table 2. Inheritance pattern of seed pericarp color in the cross among purple pericarp rice 'Heugnambyeo' and white pericarp rice 'Hwayongbyeo', 'Ishikari', and 'Ilpoombyeo'

Cross	F_1 phenotype	F_2 segregation					χ^2 (9:3:4)	p-value
		Number	Purple	Brown	White	Total		
Heugnambyeo/ Hwayongbyeo	Purple	Observed	56	20	33	109	1.682	0.90-0.10
		Expected	61.31	20.44	27.25	109		
Heugnambyeo/ Ishikari	Purple	Observed	46	13	20	79	0.279	0.90-0.10
		Expected	44.44	14.81	19.75	79		
Heugnambyeo/ Ilpoombyeo	Purple	Observed	63	21	22	106	1.019	0.90-0.10
		Expected	59.625	19.875	26.50	106		

Cross				Hwayongbyeo (*pbpbpppp*) White
	Heugnambyeo (*PbPbPpPp*)	Dark Purple	X	Ishikari (*pbpbpppp*) White
				Ilpoombyeo (*pbpbpppp*) White
Gamets	*PbPp*		*pbpp*	
F_1	*PbpbPppp* (Medium Purple)			
F_2	↓			

Gamets	*PbPp*	*pbPp*	*Pbpp*	*pbpp*
PbPp	*PbPbPpPp*	*PbpbPpPp*	*PbPbPppp*	*PbpbPppp*
	Dark Purple	Dark Purple	Medium Purple	Medium Purple
pbPp	*PbpbPpPp*	*pbpbPpPp*	*PbpbPppp*	*pbpbPppp*
	Dark Purple	White	Medium Purple	White
Pbpp	*PbPbPppp*	*PbpbPppp*	*PbPbpppp*	*Pbpbpppp*
	Medium Purple	Medium Purple	Brown	Brown
pbpp	*PbpbPppp*	*pbpbPppp*	*Pbpbpppp*	*pbpbpppp*
	Medium Purple	White	Brown	White

Fig. 1. Genetic analysis of crosses between black rice 'Heugnambyeo' and three types of white rice. The genotype might be *PbPbPpPp* for 'Heugnambyeo' and *pbpbpppp* for 'Hwayongbyeo', 'Ishikari', and 'Ilpoombyeo'.

Cross	Kewha (*PbPbPpPp*)	X	Kumgangbyeo (*pbpbPpPp*)
	Dark Purple		White
Gamets	*PbPp*		*pbPp*
F_1	*PbpbPpPp* (Dark Purple)		
F_2	↓		

Gamets	*PbPp*	*pbPp*
PbPp	*PbPbPpPp*	*PbpbPpPp*
	Dark Purple	Dark Purple
pbPp	*PbpbPpPp*	*pbpbPpPp*
	Dark Purple	White

Fig. 2. Genetic analysis of a cross between black rice 'Kewha' and white rice 'Kumgangbyeo'. The genotype might be *PbPbPpPp* for 'Kewha' and *pbpbPpPp* for 'Kumgangbyeo'.

value, 0.90-0.10, Table 2). With 2 degrees of freedom, the 10% critical value is 4.605, which is greater than the computed value of 1.682; accordingly, the hypothesis of gene segregation was not rejected. The F_2 population from the black rice 'Heugnambyeo' crossed with the white rice 'Ishikari', and 'Heugnambyeo' crossed with the white rice 'Ilpoombyeo' were also segregated at a 9:3:4 (purple: brown: white) ratio (Table 2). These findings indicate that the genotype of 'Heugnambyeo' might be *PbPbPpPp* and that of the three white pericarp rice varieties, 'Hwayongbyeo', 'Ishikari', and 'Ilpoombyeo', might be *pbpbpppp* (Fig. 2). Interestingly, seeds from F_1 plants were less purple than those from the 'Heugnambyeo' parents, and the F_2 progeny showed varying intensities of purple (Fig. 1A). Finally, the purple-colored F_2 progeny had a segregation ratio of 2 medium purple: 1 dark purple (Table 3; Fig. 1A).

Another set of crosses between purple pericarp rice and white pericarp rice was examined (Table 4; Fig. 3B). The pericarp color of all F_2 seeds from F_1 plants produced from the cross between the purple pericarp rice 'Kewha' as a pollen receptor and the white perciarp rice 'Kumgangbyeo' as a pollen donor produced a dark-purple phenotype that was the same as the pollen receptor parent, reflecting the dominant nature of the purple pericarp phenotype over white. Among 274 F_2 plants studied, the seed pericarps of 210 plants were dark-purple, like the female parent, while those of 64 plants were white, like the male parent (Table 4). We further analyzed the best-fitted data using the Chi-square (χ^2) test to test the goodness of fit for numbers of genes segregating in the population. Based on segregation in the F_2 and F_3 generation, the purple phenotype of the seed pericarps was determined to be dominant, as it segregated according to a Mendelian ratio of 3:1 (dark purple pericarp: white pericarp) (Table 4). Genetic segregation analysis indicated that two complementary genes, *Pb* and *Pp,* controlled the purple pericarps of rice and segregation of the F_2 generation at a ratio of 9 purple: 3 brown: 4 white as was observed in the three crosses of 'Heugnambyeo' with three different white pericarp rice (Table 2; Fig. 1A). However, no brown pericarps were noted in the F_2 and F_3 segregation generations produced by the cross between 'Kewha' and 'Kumgangbyeo' (Table 4; Fig. 1B). In this cross, only the *Pb* gene segregated at a ratio of 3 dark purple: 1 white. This purple pericarp segregation of 3 dark purple: 1 white is possible when both parents have homozygous dominant *PpPp* genes; therefore, the genotype of *O. sativa* L. *japonica* var. 'Kewha' might be *PbPbPpPp* and that of *O. sativa* L. *indica* var. 'Kumgangbyeo' might be *pbpbPpPp* (Fig. 3). This cross also indicated that at least one dominant *Pb* gene was necessary for the colored pericarps of rice.

Table 3. Inheritance pattern of pericarp color intensity among purple pericarp F_2 progenies followed 2 medium purple: 1 dark purple pericarp

Cross	F_2 segregation				χ^2 (2:1)	p-value
	Number	Medium Purple	Dark Purple	Total		
Heugnambyeo/ Hwayongbyeo	Observed	42	14	56	1.752	0.90-0.10
	Expected	37.33	18.67	56		
Heugnambyeo/ Ishikari	Observed	35	11	46	1.834	0.90-0.10
	Expected	30.67	15.33	46		
Heugnambyeo/ Ilpoombyeo	Observed	48	15	63	2.571	0.90-0.10
	Expected	42	21	63		

Table 4. Chi-square (χ^2) analysis for segregation of seed pericarp color of the cross between purple pericarp rice 'Kewha' and white pericarp rice 'Kumgangbyeo' at the F_2 and F_3 generations

Cross	F_2 segregation				χ^2 (3:1)	p-value
	Number	Purple	White	Total		
Kewha/Kumgangbyeo	Observed	210	64	274	0.394	0.90-0.10
	Expected	205.5	68.5	274		
	F_3 segregation				χ^2 (3:1)	p-value
	Number	purple	white	Total		
F_2-13(self)	Observed	88	30	118	0.011	0.95-0.90
	Expected	88.5	29.5	118		

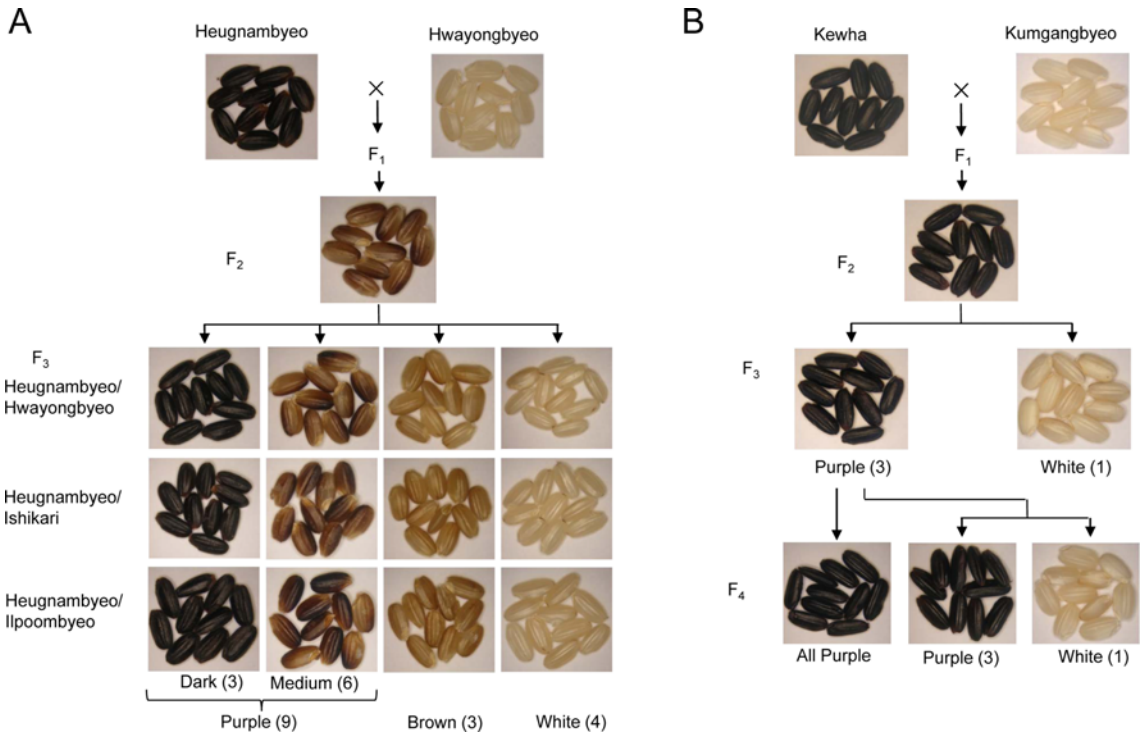

Fig. 3. Segregation analysis of pericarp color of black rice. (A) Purple pericarp rice *O. sativa* L. japonica var. 'Heugnambyeo' was crossed with three types of white pericarp rice. F_1 plants produced purple pericarps. Further selfing of F_2 plants produced F_3 seeds with purple pericarps, brown color pericarps, and white pericarps. (B) Phenotypes of the pericarps of cross materials. The purple pericarp rice 'Kewha' was crossed with white-colored pericarp wild-type 'Kumgangbyeo' rice. The F_2 seeds resulting from the F_1 plant are indicated. F_2 and F_3 populations were segregated as indicated by the arrows.

The *Pp* Allele was Incompletely Dominant Over the pp Allele

As shown in figs. 1, 2 and 3, the *Pp* gene was responsible for the seeds of the F_1 plants, and the F_2 progeny produced purple pericarps of varying intensity. The colored pericarp progeny of the 'Hugnambyeo' crosses followed a segregation ratio of 1 dark purple (*PpPp*): 2 medium purple (*Pppp*): 1 brown (no purple color) (*pppp*) (Table 2 and 3; Fig. 1 and 3A). The colored pericarp progeny of the 'Kewhabyeo' crosses were all purple (*PpPp*) (Table 4 and Fig. 2 and 3B). Therefore, the genetic configuration between *Pp* and *pp* alleles indicated that the dominant *Pp* allele was an incomplete dominant over the recessive *pp* allele.

A Dominant *Pb* allele was Essential to Color Development in the Rice Pericarp

Genotype analysis of the F_3 progeny of the crosses between purple pericarp rice and white pericarp rice was performed (Fig. 4). The 1.2 kb fragments of the *Pb* gene were successively produced in the F_3 plants. The DNA sequences of amplified DNA fragments of the *Pb* gene were exactly a part of the *Ra* gene in rice chromosome 4. The 2 bp(GT) deletion or insertion was identified in the PCR amplified

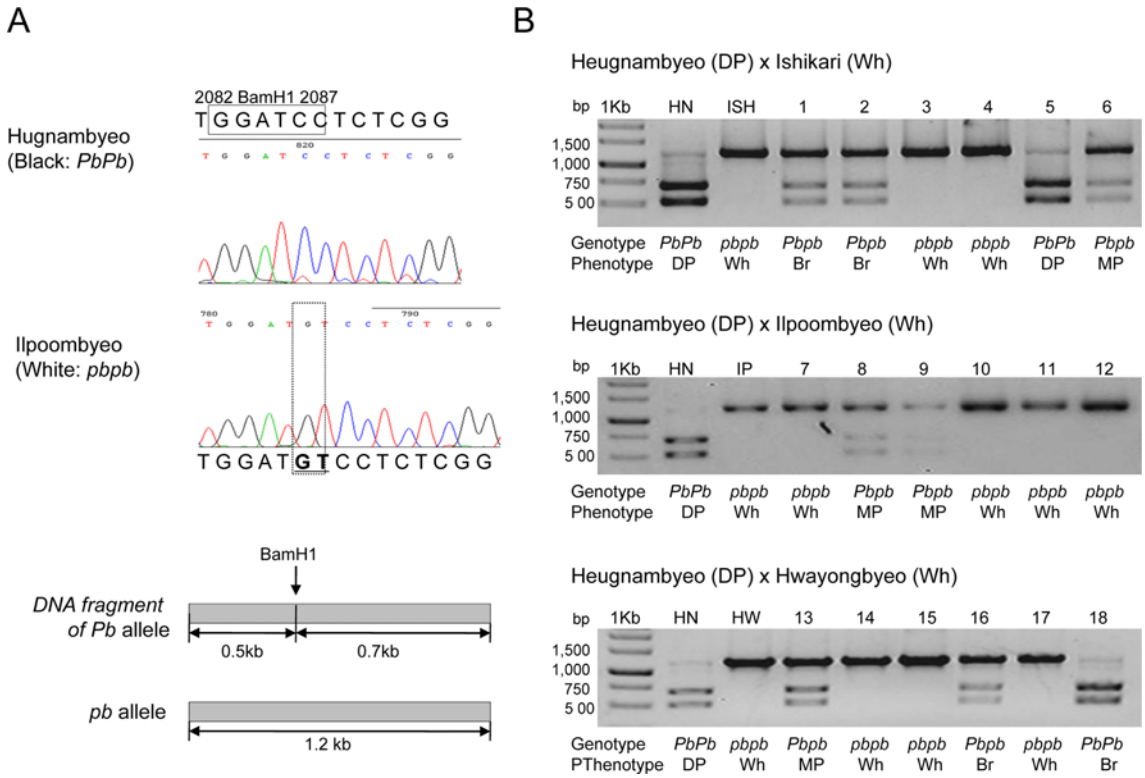

Fig. 4. Analysis of the allelic polymorphism among progeny of the crosses between a black pericarp rice and a white pericarp rice. (A) DNA sequence fragments of the amplified *Ra* gene (accession number U39860), which is the same as the rice *Pb* gene. The black pericarp rice 'Hugnambyeo' was composed of two homologous dominant *Pb* alleles and presented a *Bam*H1 restriction enzyme site in the amplified DNA sequences of each corresponding allele. The white pericarp rice 'Ilpoombyeo' was composed of two homologous recessive *pb* alleles without any *Bam*H1 restriction enzyme site in the amplified DNA sequences of the *pb* allele. The *pb* allele of the white pericarp rice has a 2bp (GT) insertion in the BamH1 site of the same DNA sequences in the *Pb* allele. Schematic drawings are presented after *Bam*H1 digestion in the DNA of the *Pb* allele resulting in division into two fragments of DNA. (B) Genetic analysis of the genotypic and phenotypic constitutions of the F_3 progeny of the crosses between a black pericarp rice and a white pericarp rice. Genotypes are *PbPb* for homozygous dominant alleles, *Pbpb* for heterozygous alleles, and *pbpb* for homozygous recessive alleles. Phenotypes are indicated by the pericarp colors, with DP indicating dark purple, MP indicating medium and mixed purple, Wh indicating white, and Br indicating brown.

DNA sequences from genomic DNA of the purple rice 'Hugnambyeo' and the white pericarp rice 'Ilpoombyeo' (Fig. 4A). Further DNA sequence analysis of the parental lines indicated that all of the PCR fragments in the purple rice showed the 2 bp(GT) deletion, but that those of white rice showed the 2bp(GT) insertion. These results were the same as those reported in an analysis of the *Pb* gene by Wang and Shu (2007), confirming that the rice *Ra* and *Pb* genes are the same gene. The area of the GT deleted sequence of the dominant *Pb* allele offered a 5'-GGATCC sequence, which can be utilized as a *Bam*H1 restriction enzyme site. Therefore, *Bam*H1 restriction digestion of the 1.2 kb PCR products of purple rice produced two DNA fragments, while those of white pericarp rice did not. As shown in figure 4, genotype analysis based on PCR and *Bam*H1 digestion showed that all dark purple pericarp progeny presented two bands of digested fragments, indicating that the genetic constitutions of these plants were the homozygous dominant *PbPb* alleles. All medium purple progeny presented three bands, with one band of 1.2 kb and two bands of the digested fragments in the agarose gel, indicating that these plants had heterozygous constitutions of *Pbpb* alleles. Interestingly, the brown rice showed two different genotypes, one that presented three bands on the agarose gel, indicating heterozygous *Pbpb* alleles, and another that showed two *Bam*H1 digested bands, indicating homozygous dominant *PbPb* alllels (Fig. 4B). However, all white progeny presented only one band of 1.2 kb in the agarose gel, indicating that the genetic constitutions of the white pericarp rice plants were composed of homozygous recessive *pbpb* alleles. Overall, at least one dominant and functional *Pb* allele was present in both purple and brown pericarp rice. Therefore, the presence of at least one dominant *Pb* allele is an essential factor for color development in rice pericarps.

The Content of Cyanidin-3-*O*-glucoside was Determined by the Number of Dominant *Pp* Alleles

Extracts from the dark purple, medium purple, brown, and

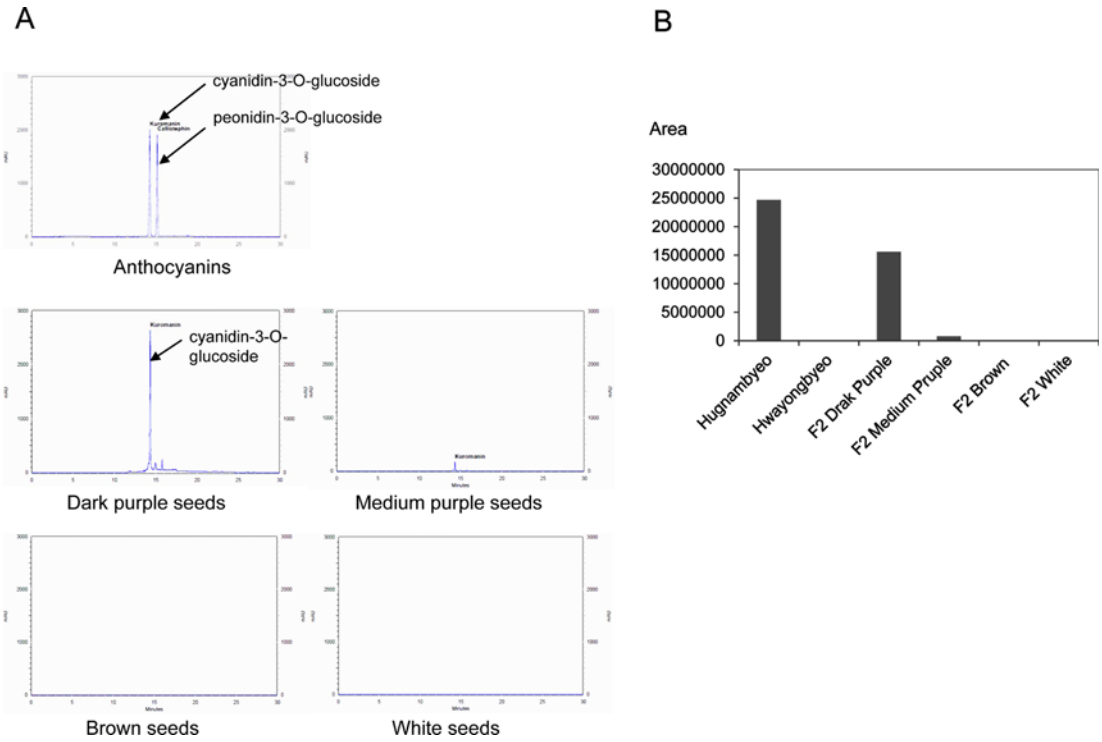

Fig. 5. Anthocyanin profile of progeny of black rice and white rice extracts analyzed by HPLC. (A) HPLC profiles of extracts from the purple, brown or white pericarps of rice seeds from the F_2 progeny. The first peak is cyanidin-3-*O*-glucoside (kuromanin) and the second peak is peondin-3-*O*-glucoside (callistephin) in standard. Retention time is shown on the horizontal line and the amount of absorption unit (mAU) is shown on the vertical line. (B) Measurement of cyanidin-3-*O*-glucoside in each pericarp of rice seeds. Arbitrary numbers of absorption are shown on the vertical line. A high amount of cyanidin-3-*O*-glucoside was detected in the 'Hungnambyeo' (*PbPbPpPp*) and the dark purple seeds (*Pb_PpPp*) of the F_2 progeny. However, a relatively small amount of cyanidin-3-*O*-glucoside was detected in the medium purple seeds (*Pb_Pppp*) of the F_2 progeny. The 'Hwayongbyeo' (*pbpbpppp*) and the brown (*Pb_pppp*) or white pericarp seeds (*pbpbpppp*) of the F_2 progeny were not detected in the cyanidin-3-*O*-glucoside.

white bran of seed pericarps were subjected to HPLC analysis (Fig. 5). A strong peak of cyanidin-3-*O*-glucoside (kuromanin) was observed in the dark purple pericarp rice of the 'Heugnambyeo', 'Kewha', and F_2 progeny, which harbor the *Pb_PpPp* genotypes. There was no detectable peak in the white pericarp rice, including the 'Hwayongbyeo', 'Ishikari', 'Ilpoombyeo', and 'Kumgangbyeo' variants. Moreover, no anthocyanin peak was identified in the bran extraction of the brown pericarp rice (*Pb_pppp*), such as the F_2 progeny of 'Heugnambyeo' crossed with white pericarp rice. Anthocyanin was detected in the medium purple pericarp rice (*Pb_Pppp*) of the F_2 progeny, but the cyanidin-3-glucoside content was significantly lower than that of the dark purple variant (*Pb_PpPp*) (Fig. 5). The biochemical phenotypes of the progeny showed that the level of cyanidin-3-*O*-glucoside was determined by the number of copies of *Pp* alleles of the genotype in each progeny.

Discussion

The inheritance pattern of purple pericarps in rice was studied in crosses of pollen receptors from the black rice 'Heugnambyeo' and three pollen donors from plants with white pericarps. All F_1 plants produced purple pericarp seeds, indicating that the purple pericarp characteristic of rice dominates over the white pericarp characteristic. χ^2 analysis of F_2 segregation data fit ratios of 9:3:4 rather than the Mendelian dihybrid ratio of 9:3:3:1. The pericarp color in black rice was determined by two dominant, complementary genes, *PbPp* for purple, *Pbpppp* for brown, and *pbpbPpPp* or *pbpbpppp* for white. The *Pb* gene alone is responsible for the accumulation of pigment in the pericarps of brown grains. Furthermore, purple pericarp rice grains require the *Pp* gene (Wang and Shu 2007; Wang et al. 2009). As observed in this study, both alleles of the *Pb* and *Pp* genes are involved in the purple pigmentation of rice pericarps. Alleles of homozygous recessive *pbpb* genes are non functional in the presence of the dominant *Pp* gene, resulting in the recessive epistasis interaction. This recessive epistasis was previously observed in wheat pericarp inheritance, and it is known that two complementary dominant genes (*Pp1* and *Pp3*) control the deposition of purple pigmentation in wheat pericarps (Dobrovolskaya et al. 2006; Khlestkina et al.

2010). Due to recessive epistasis, the results of the crosses between the black rice 'Heugnambyeo' and the white pericarp rice strains 'Hwayoungbyeo', 'Ishihikari' and 'Ilpumbyeo' were consistent with the modified Mendelian dihybrid ratio of 9:3:4 (purple: brown: white). Based on this phenotype analysis, we conclude that the genotype of purple pericarp rice variant 'Heugnambyeo' was *PbPbPpPp* and the three white pericarp rice varieties were *pbpbpppp* (Fig. 2). Interestingly, the F_2 progeny of the crosses among pollen receptors from 'Heugnambyeo', which has purple pericarps, and three pollen donors from plants with white pericarps did not show the same intensity of purple (Fig. 1A). The seeds of the F_1 plant seeds and the medium purple seeds of the F_2 plants showed purple color deposition in a brown background. The *Pb* gene in the absence of *Pp* produces a brown-colored grain, which suggests that the *Pp* gene acts in a complementary fashion with the *Pb* gene to increase the content of the pigment from brown to purple.

As shown in fig. 2 and 3A, the purple pericarp progeny followed a segregation ratio of 1 dark purple (*PpPp*): 2 medium purple (*Pppp*): 1 brown (*pppp*), suggesting that the dominant *Pp* allele was incomplete over the recessive *pp* allele of the *Pp* gene (Table 3). As shown in figure 5, the segregation pattern of the *Pp* gene was correlated with the amount of anthocyanin in the segregation of 1 dark purple (*PpPp*): 2 medium purple (*Pppp*). Moreover, the dark purple rice grains (*PpPp*) contained relatively higher amounts of cyanindin-3-*O*-glucoside than the medium purple seeds (*Pppp*) of the F_2 progeny. Based on the genotype analysis shown in figure 4, the pericarp color phenotypes of the F_3 progeny matched the genotypes exactly (Fig. 1). We also demonstrated that the DNA fragments of the *Pb* gene in the purple rice showed a 2 bp (GT) deletion when compared to that of the white rice (Fig. 4). These results were identical to those of a *Pb* gene analysis conducted by Wang and Shu (2007). Here, we also confirmed that the *Pb* gene in rice chromosome 4 is the *PURPLE PERICARP B* gene. In this genotype analysis, progeny with heterozygous *Pbpb* alleles showed two different phenotypes, medium purple pericarps (MP) and the brown pericarps (Br). Furthermore, the progeny of dominant homozygous *PbPb* alleles had dark purple (DP) or brown pericarps (Br), while the progeny of homozygous recessive *pbpb* alleles produced white pericarps (no color deposition) (Fig. 4). These findings suggest that the presence of at least a dominant and functional *Pb* allele was essential to color development in rice pericarps. In addition, we demonstrated that the purple anthocyanin color deposition was not determined by the *Pb* gene. Although genotype analysis of the *Pp* gene of the progeny was not performed in this study, it is clear that the *Pp* gene determines the level of cyanidin-3-*O*-glucoside deposition in black rice.

In this genetic analysis, we demonstrated that the *Pb* and *Pp* genes are involved in purple pigmentation of rice pericarps with the epistatic interactions. Furthermore, we showed that the dominance of the *Pp* allele over the recessive *pp* allele is incomplete. Therefore, deposition of the cyanidin-3-glucoside in black rice is affected by the *Pp* gene.

Materials and Methods

Plant Materials

Several black and white rice germplasms were grown in a paddy field of Yeungnam University, Gyeongsan, Korea. The purple pericarp rice used were *Oryza sativa* L. *japonica* var. 'Kewha' and *O. sativa* L. *japonica* var. 'Heugnambyeo', while the white pericarp rice *O. sativa* L. *japonica* var. 'Hwayongbyeo', *O. sativa* L. *japonica* var. 'Ishikari', *O. sativa* L. *japonica* var. 'Ilpoombyeo', and *O. sativa* L. *indica* var. 'Kumgangbyeo' were used as wild-type controls.

Phenotypic Analysis and Agronomic Data Scoring

The pigmentation in the pericarps was documented and photographed using a digital camera. The phenotypes of the purple pericarp plants and wild-type white pericarp plants were also recorded (Matin and Kang 2012). 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. Twenty-five-day-old seedlings were transplanted as single plants in the experimental paddy field and agronomic data from vegetative to reproductive growth periods were recorded. Specifically, 15 plants of each accession were evaluated for each type of agronomic data considered. Additionally, the average number of tillers per plant was calculated based on data obtained from 15 plants. The average numbers of spikelets were recorded from the five plants using five panicles from each plant. The spikelet fertility percentage was scored as the number of filled grains divided by the number of total spikelets from each panicle. 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. The mean number of days to heading of individuals was taken as the heading date. The days to heading were then converted from the day of transplantation to obtain the mean heading date. For grain weight, 100 ripped spikelets were dehulled and the weight in grams was measured using an electronic balance.

Genetic Analysis

Genetic studies were conducted to analyze the inheritance pattern of the pericarp color of rice. We visually assessed the pericarp color of matured seeds from the F_1 and F_2 populations for individuals with purple, brown or white seed pericarps. To evaluate the inheritance pattern of purple pericarps, segregation analysis of the purple pericarps was carried out using F_1 and a large population of F_2 progeny from crosses among *Oryza sativa* L. *japonica* var. 'Heugnambyeo' with purple pericarps as a pollen receptor, and *O. sativa* L. *japonica* var. 'Hwayongbyeo', *O. sativa* L. *japonica* var. 'Ishikari', and *O. sativa* L. *japonica* var. 'Ilpoombyeo' with white pericarps as pollen donors. A total of 20 fertilized seeds for each cross were obtained and the resultant F_1 seeds were grown in the field to produce F_1 plants. The F_1 plants were then allowed to self-fertilize to produce F_2 seeds, which

were collected from a single F_1 plant and grown in the field under natural conditions. The phenotypic data of the F_2 segregations were documented, and F_3 seeds from a single panicle were harvested separately from each F_2 plant at the mature stage. Segregation analysis of pericarp color was also conducted using F_1 and a large population of F_2 and F_3 progeny from the cross between *O. sativa* L. *japonica* var. 'Kewha' with purple pericarps as a pollen receptor (P_1) and *O. sativa* L. *indica* var. 'Kumgangbyeo' with white pericarps as a pollen donor (P_2). The genotype of the parents was determined using the seed pericarp phenotype of the F_1, F_2 and F_3 populations. Genomic DNA was extracted from leaf tissues using the CTAB buffer method (Matin and Kang, 2012). Determination of allelic differences in *Pb* genes among the progeny of black pericarp rice and white pericarp rice crosses was performed based on PCR-based polymorphism of the *Ra* gene, which is a homologue of the *Pb* gene (Hu et al., 1996; Wang and Shu 2007). Briefly, polymerase chain reaction (PCR) using primers for the transcriptional activator *Ra* gene (accession number U39860) was performed (forward primer 5'-GGGAGAAGCTCAA-CGAGATG and reverse primer 5'-GGGTGGCAGATTCATCACTT). The PCR amplified fragments were then sequenced to define the *Pb* gene. For genotype analysis, the PCR products were digested with *Bam*H1 restriction enzyme and run on 1.2% agarose gels.

HPLC Analysis of Rice Seed Extract

Ten grams of rice pericarp powder were extracted with 50 mL of 70% ethanol as an extraction solvent for 24 h at 25°C in the dark. 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 (4.6×250 mm; Sheseido) at 30°C with the detection absorbance set at 520 nm. The elution system consisted of 5% formic acid (solvent A) and 5% acetonitrile containing 5% formic acid (solvent B). Elution was conducted using a linear gradient of B into A at a flow rate of 1.0 mL/min as follows: elution starting with 0-35.5% B at 0-23 min under isocratic flow and then increasing from 35.5-100% B at 24-45 min. Kuromanin and Callistephin (Sigma, St. Louis, USA) were used as standard chemicals for measurement of cyanidin-3-*O*-glucoside and pelagonidin-3-*O*-glucoside, respectively.

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