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Studies on the effects of pericarp pigmentation on grain development and yield of black rice

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Abstract

Black rice was characterized by dark purple pericarp and much lower yield than that of white rice varieties. We investigated the factors responsible for the lower yields of black rice by conducting crosses of rice with purple pericarp mutant and white pericarp cultivars. The seed weight of black rice was lower than that of brown or white rice progeny within the crosses. The F_2 segregants showed increased anthocyanin deposition with simultaneous reduction of chlorophyll in the seed pericarp. These results revealed that an unknown metabolic alteration exists in the chlorophyll synthesis due to the high level of cyanindin-3-0-glucoside deposition in the purple pericarp rice, indicating that anthocyanin deposition in the pericarp of black rice hindered the photosynthetic rate, leading to low yield. These findings suggest that the low yield of black rice is closely related to physiological factors other than the genetic yield traits.

Key words: *Oryza sativa*, cyanidin-3-O-glucoside, pericarp color development, *Prp* traits

Introduction

The outermost layer of husked rice, which is known as the pericarp, is located closely beneath the hull of rice grain. Most of the cultivated rice possesses white colored pericarps; however, some varieties can also produce grain with purple, red, green or brown pericarp (Reddy et al. 1995; Kang et al. 2006; Rahman et al. 2013). Black rice is characterized by purple pericarps, which require two genes, purple pericarp *A* (also known as *Pp, Prpa* and *Prp1*), and purple pericarp *B* (also known as *Pb, Prpb* and *Prp2*) located on chromosomes 1 and 4, respectively (Wang and Shu 2007; Wang et al. 2007). Two dominant complementary genes control the rice pericarp colour, *Pb_Pp_* for purple and *Pb_pppp* for brown, whereas *pbpbPp_* or *pbpbpppp* result in white pericarps (Hsieh and Wang 1964). The intensity of purple colour was also recently confirmed to depend on the number of dominant *Pp* alleles on chromosome 1 (Rahman et al. 2013).

In black rice, high levels of anthocyanins accumulate in the pericarp during the seed development stages, resulting in its dark purple colour (Reddy et al. 1995; Abdel Aal et al. 2006). There are two major anthocyanin pigments in rice grains, cyanidin-3-0-glucoside (C3G) and peonidin-3-0-glucoside (P3G) (Nam et al. 2006). Rice anthocyanins have received significant attention as health promoting functional food ingredients owing to their long established biomedical properties, which include anti-cancer activity, anti-oxidant activity and anti-inflammatory effects (Hyun and Chung 2004; Nam et al. 2006). However, the yield potential of black colored rice is much lower than that of white rice (Zhang et al. 1994) and farmers are often not willing to cultivate colored rice. Therefore, it is necessary to find out the unnoticed reasons responsible for lower yield of black rice. Additionally, the pigment deposition patterns during the grain filling stage and its influence on yield have not been explained properly. Therefore, we investigated chlorophyll and anthocyanin pigments deposition in the pericarps of seed development and suggested their possible effects on different physiological and agronomic traits leading to the low yield of colored rice.

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Materials and methods

Rice (*Oryza sativa* L. *japonica*) with purple pericarp was crossed with three different rice cultivars having white pericarps. Among the selected cultivars, the purple pericarp rice 'Heugnambyeo' was used as the pollen receptor, whereas the white pericarp rice 'Hwayongbyeo', 'Ishikari' and 'Ilpumbyeo' were used as the pollen donors. The F_1 plants were allowed to self-fertilize to produce F_2 seeds. The F_2 population in each cross had 200 plants. The phenotypic and physiological data describing the progenies were documented, and grains from a single panicle were harvested separately from each F_2 plant at the mature stage for chemical analysis.

Analysis of different traits

Agronomic data were recorded from rice in the vegetative and reproductive stages and during postharvest. Different phenotypic traits of purple pericarp, brown pericarp and white pericarp rice plants were also recorded according to Matin et al. (2010).

The chlorophyll contents of the flag leaves and grains were analyzed. The grain filling rate, moisture and anthocyanin contents in grains were investigated. In all physiological analyses, three flag leaves from the middle and grains from the top three primary branches of panicles were used. Initially, each plant was tagged at fertilization stage. Samples were collected every five days from day 5 to 35 after fertilization.

Measurement of chlorophyll content

Total chlorophyll was determined using the formula described by Porra (2002). Briefly, the leaves (0.5 g) were placed in a mixed solution (10ml) of acetone and absolute ethyl alcohol (volume ratio 2:1) and extracted by shaking over night using an electric horizontal shaker. The optical densities (OD) of the extracts at 645 and 663 nm were then measured using a T60 UV-visible spectrophotometer to determine the content of chlorophyll. The chlorophyll content of husked grain was also measured by same method.

Grain filling rate and measurement of moisture content

Spikelets from upper three primary branches of panicles were collected and husked. The fresh weight of 100 grains was measured. The grain was dried at 105°C for 1 h and then kept at 80°C until they reached constant weight. The dry weight of those grains was measured. The grain filling rate and the grain moisture content were determined using the following formula (Ji et al. 2012):

$$\text{Grain filling rate (GFR)} = \frac{W_2/N_2 - W_1/N_1}{t_2 - t_1}$$

$$\text{Grain moisture content (GMC)} = \frac{G-W}{G}$$

where, G is the fresh weight of the grains, W is the dry weight of the grains; t_1 is number of days after fertilization of previous sample collection, t_2 is number of days after fertilization at GFR measuring date and N is the number of grains.

Measurement of anthocyanin content

The anthocyanin content in husked grains was measured according to the procedures described previously with slight modification (Huang et al. 2006). Briefly, 1 g of seeds was extracted three times using a solution of 15 mL HCL-methanol (0.15% HCl/methanol, 15: 85) for 4 h. The extract was then filtered and its absorbance at 535 nm was measured using a T60 UV-visible spectrophotometer to determine the anthocyanin content determined as follows:

$$\text{Anthocyanin content} = A \times MW \times DF \times 100/(\varepsilon \times W)$$

where, A is the absorbance, MW is the molecular weight of cyanidin-3-0-glucoside chloride ($C_{21}H_{21}ClO_{11}$, 484.84 Da), DF is the dilution factor, ε is the molar absorptivity (34 300), and W is the sample weight (g).

HPLC analysis of rice bran extract

Rice pericarp powder was made by a rice-polishing machine. Ten grams of rice pericarp powder was extracted with 50 ml of 70% ethanol for 24 h at 25°C in the dark. The extracts were then centrifuged at 10,000 × g for 20 min, after which they were filtered through a 0.25 µm PVDF filter (Millipore, Billerica, MA, USA). Finally, the filtered samples (10 µl) were injected into an HPLC (high performance liquid chromatography) system (Sheseido, Tokyo, Japan) equipped with a CAP CELL PAK C18 column (4.6 x 250mm; Sheseido) at 30°C and an UV detector 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: 0-35.5% B from 0-23 min isocratic, then

35.5–100% B from 24-45 min. Kuromanin and Callistephin (Sigma, St. Louis, MO, USA) were used as standard chemicals for cyanidin-3-0-glucoside and peonidin-3-0-glucoside, respectively.

Results

Pigment accumulation in rice pericarps during grain development

Pigment accumulation at different developmental stages is depicted in Fig. 1. Accumulation of green pigments in the seed pericarps of white rice started after fertilization and reached the maximum level during the dough stage (7-14 DAP) of seed development, after which the pigments decreased until none were observed in the mature seeds of white rice. In contrast, seeds of colored pericarp rice exhibited green pigment deposition after fertilization to the milking stages (3-6 DAP), followed by the disappearance of green pigment from 10 DAP (days after pollination). The levels of chlorophyll contents did not differ significantly from those observed in brown rice whereas, while the lowest chlorophyll content was observed in black rice at 10 DAP and 15 DAP (Fig. 2A). In brown pericarp rice, brown pigment deposition started at 15 DAP, after which the entire seed became brown and green pigment completely disappeared at 25 DAP. In purple pericarp rice, purple pigment accumulation occurred near the apex of the seed at the beginning of dough stage and persistently increased until physiological maturity (21-25 DAP), after which it decreased until harvest. The anthocyanin content of pericarp powder of black rice was much higher than that of brown and white rice throughout the growth season of rice (Fig. 2B). A gradual increase in anthocyanin content from day 5 to 25 after pollination was observed in purple pericarp rice (Fig. 2B). Furthermore, the maximum anthocyanin content of purple pericarp rice was attained at 25 DAP, after which it gradually decreased until harvest. Finally,

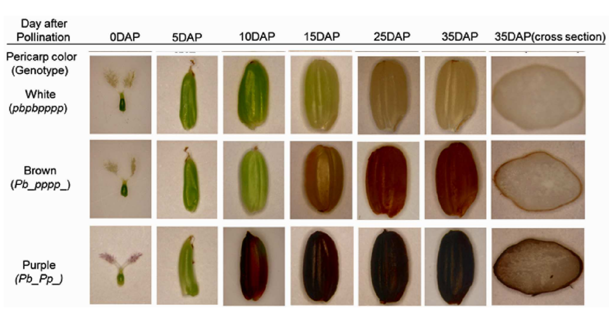

Fig. 1. Progressive seed developmental stages. Close-up images were taken at 0.67× magnification

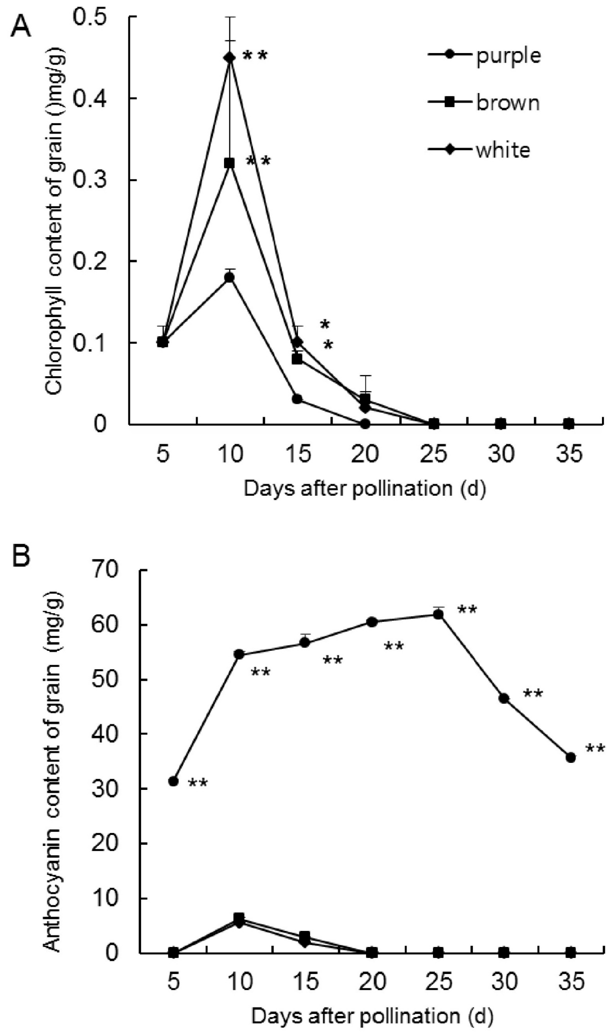

Fig. 2A&B: Changes in chlorophyll and anthocyanin contents of seed extracts from the purple, brown and white pericarp rice. A = Changes in grain chlorophyll content. B = Changes in grain anthocyanin

the endosperms of black, brown and white rice became faded purple, faded brown and white, respectively (Fig. 1). A strong and identical peak of cyanidin-3-O-glucoside was found in pericarp extract of 'Heugnambyeo' parent rice and purple pericarp F_2 progeny, while trace amounts of peonidin-3-O-glucoside were found in both rice as well. In contrast, no anthocyanin was detected in the pericarp extract of white and brown pericarp progeny (Fig. 3).

Anthocyanins deposition in seed pericarp decreased in 100 seed weight

F_1 hybrids obtained from the crossing of black rice with three different white rice cultivars exhibited high

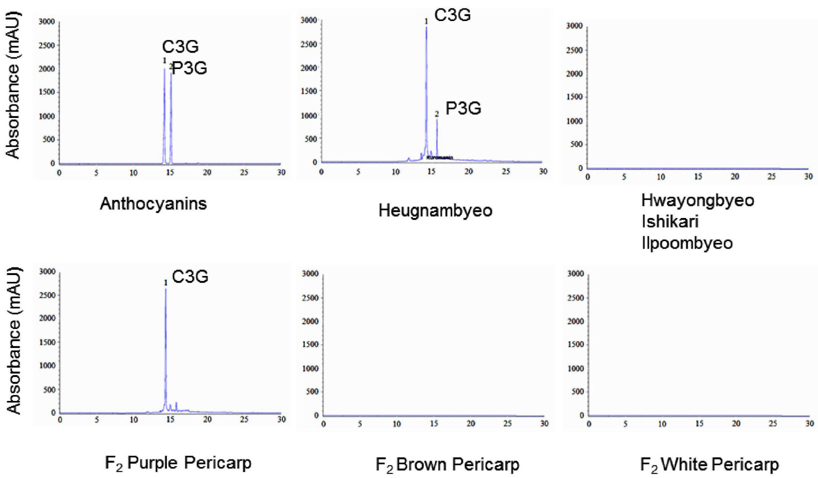

Fig. 3. Anthocyanins profile of mature rice pericarp extract analyzed by HPLC. The first peak is cyanidin-3-O-glucoside and the second peak is peondin-3-O-glucoside in standard

levels of variation in vegetative and reproductive traits (Table 1). Specifically, the plant height of hybrid rice varied from 113.7 cm to 119.8 cm, with most hybrid plants showing higher height and tiller numbers than their parents. However, there were no significant differences in plant height, length and width of flag leaf, panicle number per plant, spikelet number per panicle, seed fertility percentage, or seed width among the F_2 population within the crosses (Table 2). Though, among the F_2 plants the chlorophyll content of flag leaves of white rice and brown rice was significantly higher at 10, 30 and 35 DAP than that of black rice (Fig. 4). Similar trends in grain-filling rates were observed among white, brown, and purple pericarp rice at all growing periods except 15 DAP. The highest grain-filling rate (1.77 mg/p.d.) was recorded in white rice, and this value did not differ significantly from those of the grain-filling rate (1.63 mg/p.d.) of brown rice; however, the lowest grain-filling rate (1.35 mg/p.d.) was recorded at 15 DAP in black rice, and this value was different from that of the other two cultivars (Fig. 5A). The

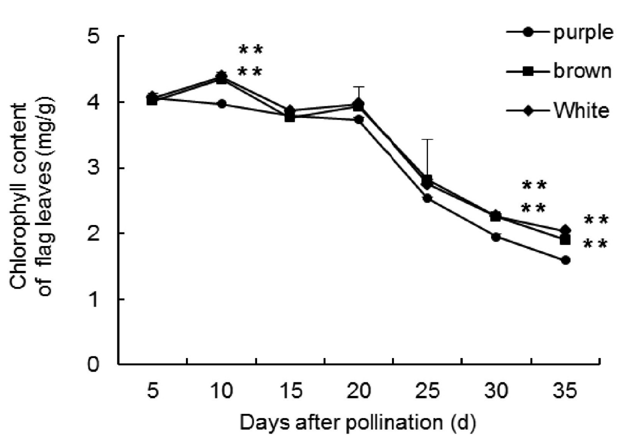

Fig. 4. Changes in chlorophyll content of the flag leaf extracts from purple, brown and white pericarp rice

Table 1. Values of agronomic traits from purple/white pericarp rice F_1 hybrids derived from field collections

F_1 hybrid	Pericarp color	Plant height (cm)	Culm length (cm)	Flag leaf length (cm)	Flag leaf width (cm)	No. of tillers/ plant	No. of panicle/ plant	Panicle length (cm)	No. of fill grains/ panicle	No. of unfill grains/ panicle	No. of grains/ panicle	Seed fertility (%)
Heugnambyeo/ Hwayongbyeo	Purple	116.7±1.4	76.9±0.6	38.0±0.8	1.46±0.06	27.1±2.0	24.4±0.6	23.0±1.0	140.1±2.3	13.5±2.0	154.7±1.5	90.32±1.8
Heugnambyeo/ Ishikari	Purple	119.4±0.8	78.9±1.1	39.0±1.8	1.34±0.07	27.9±1.8	25.3±1.0	19.6±0.5	145.7±2.5	15.8±1.6	160.1±1.2	90.63±2.0
Heugnambyeo/ Ilpumbyeo	Purple	113.7±1.3	74.9±0.8	37.0±2.3	1.38±0.05	25.3±0.7	22.5±0.8	24.2±1.1	155.2±3.3	12.3±1.5	165.2±4.3	93.93±1.7

*Data are means ±standard error of five observations for each trait

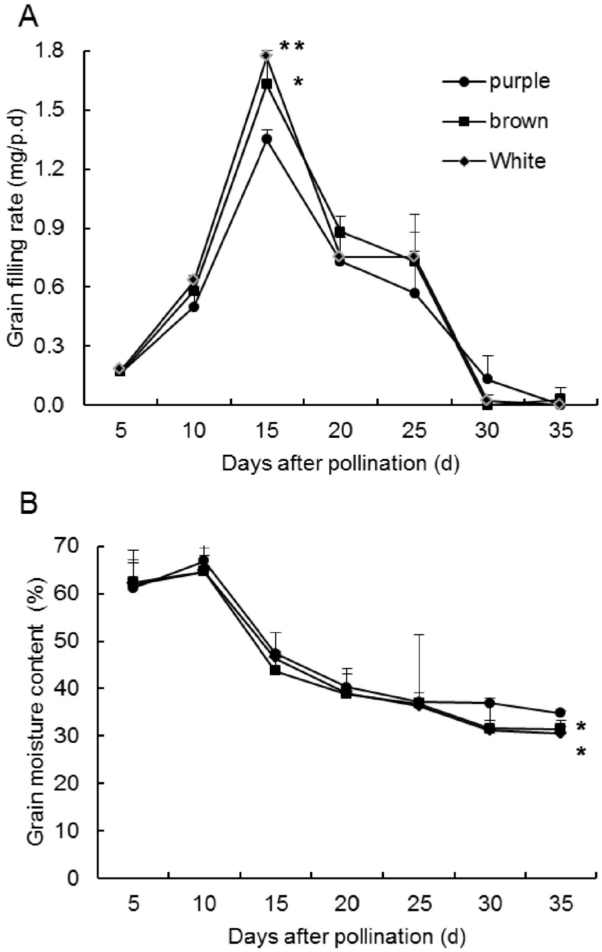

Fig. 5. Changes in grain filling rate and grain moisture content of purple, brown and white pericarp rice. (A) Changes in grain filling rate (mg/p.d.). (B) Changes in grain moisture (%)

grain moisture content did not differ among rice varieties of different color, although the purple pericarp rice showed significantly higher grain moisture content than the other colored rice at 35 DAP (Fig. 5B).

The 100 seed weight of rice with white and brown pericarps was significantly higher than that of purple pericarp rice, but no significant differences were observed between rice with white and brown pericarps (Fig. 6). The highest 100 seed weight (2.336 g) was recorded for white rice, and this value did not differ significantly from that of the 100 seed weight (2.158 g) of brown rice, whereas the lowest 100 seed weight (1.864 g) was recorded in black rice obtained from the cross between 'Heugnambyeo' and 'Hwayongbyeo' (Fig. 6A) and this value was significantly different from that of the other colored rice. Similar results of 100 seed weight were also observed from another two

Table 2. Comparative agronomic traits among purple, brown and white pericarps F_2 population derived from individual crosses

Cross	Pericarp color	Plant height (cm)	Culm length (cm)	Flag leaf length (cm)	Flag leaf width (cm)	No. of tillers/ plant	No. of panicle/ plant	Panicle length (cm)	No. of fill grains/ panicle	No. of unfill grains/ panicle	No. of grains/ panicle	Seed fertility (%)	Seed length/ width
Heugnambyeo/ Hwayongbyeo	Purple	102.8	66.0	36.8	1.24	9.8	8.4	22.7	94.0	38.8	133.3	68.64	1.80
	Brown	100.0	69.4	32.4	1.24	10.4	7.8	21.2	95.4	29.8	125.2	78.90	1.85
	White	106.8	70.8	36.0	1.36	9.2	7.0	22.8	112.8	40.2	153.0	75.75	1.85
Heugnambyeo/ Ishikari	Purple	110.8	71.4	39.4	1.22	13.4	11.0	20.8	109.4	27.2	136.6	79.03	1.91
	Brown	115.2	71.6	37.6	1.28	14.2	11.0	21.4	111.8	42.2	154.0	77.24	2.01
	White	107.8	74.2	33.6	1.22	14.0	12.8	20.6	125.8	11.6	137.4	91.61	1.97
Heugnambyeo/ Ilpumbyeo	Purple	94.0	66.8	33.8	1.22	13.0	11.0	23.1	127.6	25.2	152.8	81.53	1.80
	Brown	107.6	69.4	38.2	1.37	13.6	12.4	23.3	138.6	16.2	154.8	89.22	1.89
	White	109.0	72.4	38.6	1.38	9.8	8.6	22.8	142.2	28.6	170.8	82.83	1.91

*Each data represents mean of five observations for each trait; no significant variation was observed (Dunnett's multiple comparison test)

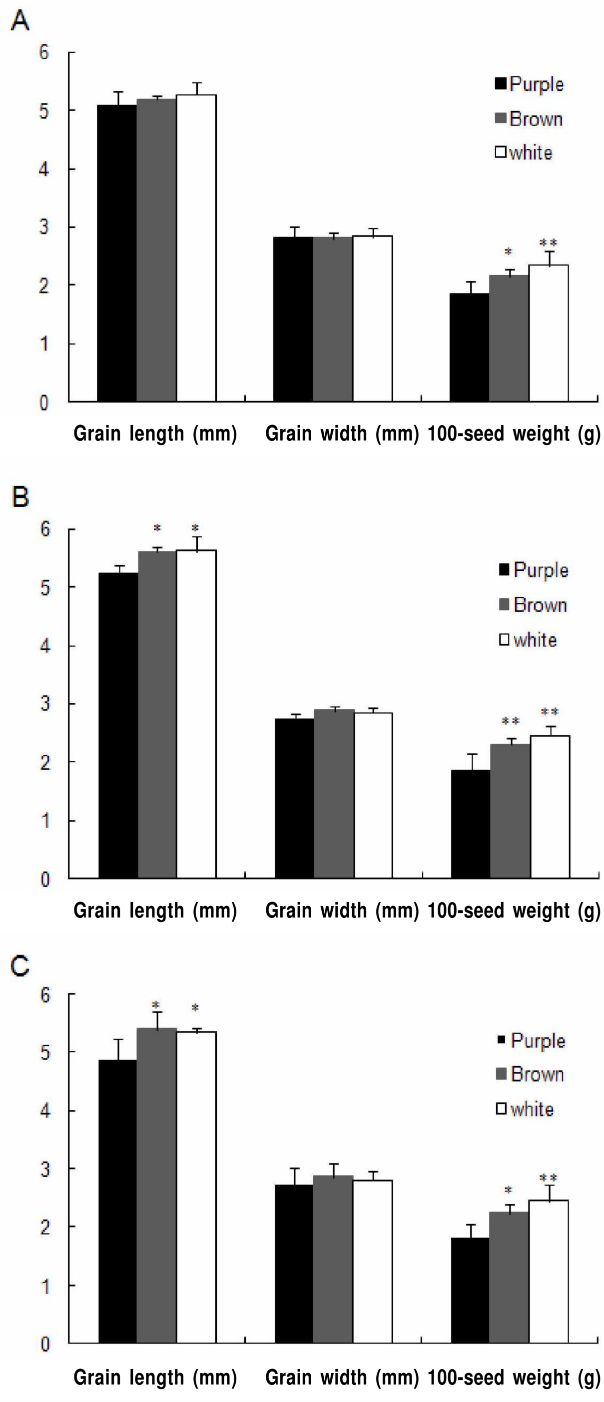

Fig. 6. Comparison of seed length (mm), seed width (mm) and 100 seed weight (g) of the purple, brown and white pericarp in F_2 population. A = Cross between Heugnambyeo and Hwayongbyeo. B = Cross between Heugnambyeo and Ishikari. C = Cross between Heugnambyeo and Ilpumbyeo. Data are the means ± S.D. of five observations of each trait; **P < 0.01; *P < 0.05 versus purple pericarp rice (Dunnett's multiple comparison test)

crosses studied. As with seed weight, the seed length of purple pericarp rice was significantly lower than that of white pericarp and brown pericarp rice generated from the cross among 'Heugnambyeo' with 'Ishikari' and 'Ilpumbyeo' Figs. 6B and 6C. No significant differences were observed in seed length among white pericarp, brown pericarp and purple pericarp rice obtained from the cross between 'Heugnambyeo' and 'Hwayongbyeo' (Fig. 6A).

Discussion

Rice seeds have a protective layer known as the hull, which covers the bran, endosperm and embryo. After removing the hull, rice is considered to be dehulled and further decoating results in milled rice, which is usually used as food. Dehulled black rice is an important source of dietary fiber, vitamins, minerals and anthocyanins that are beneficial to human health (Hyun and Chung 2004; Nam et al. 2006; Chiang et al. 2006). This is the first study to demonstrate that green pigments accumulation in pericarp of white rice started after pollination, reached the maximum level in the dough stage and then decreased until no pigments were present in the pericarps of mature seeds (Fig. 1). Green pigments accumulated from the flowering to the milking stage, after which they degraded continuously until they completely disappeared at 25 DAP (Fig. 1, 2A). When green pigment started to degrade at the beginning of the desiccation stage, other coloured pigments started to accumulate and continued to intensify until the seed reached physiological maturity. These findings are similar to those of anthocyanin pigments, which begin to accumulate coincident of chlorophyll degradation in litchi pericarp during fruit development (Lai et al. 2015). Our results revealed a gradual increase in anthocyanin content from 5 to 25 DAP (Fig. 2B), with the whole pericarp of rice seed becoming purple after 15 DAP.

The lower yield of purple pericarp rice was previously reported to be a result of variations in temperature, intrinsic physiology and agronomic traits (Zhang et al. 1994; Cai 2001; Ji et al. 2012). We also found that lower seed weight and seed length had a significant relationship and that reduced seed size resulted in decreased 100 seed weight (Ashraf et al. 1994), but the reasons behind the low yield of colored rice have not yet been clearly elucidated. In the present study, we investigated the pattern of pigment accumulation during the grain filling stage and its impact on the yield and quality of rice. We also demonstrated that purple pericarp rice accumulated relatively higher amounts of C3G and trace amounts of P3G, whereas anthocyanins were not found in white and brown pericarp rice. Our findings are similar to previous reports that high amounts of anthocyanin were deposited in purple pericarp rice and negligible amounts in brown and white pericarp rice (Rahman et al. 2013). Furthermore, rice with purple pericarp has been reported to have significantly lower seed weights than rice white pericarp (Wang et al. 2009). One possible explanation for lesser grain filling rate of purple pericarp rice could be due to partitioning of energy required for grain filling that is diverted to pigment production. These findings clearly indicate that the lower seed weight of purple pericarp rice might be due to the accumulation of anthocyanin in the pericarps. Here, we demonstrated that negative relationships clearly exist between chlorophyll and anthocyanins content in the pericarp of black rice, indicating that anthocyanin deposition leads to subsequent inhibition of chlorophylls in the chloroplasts of pericarps. It has been suggested that this occurs because the deposition of sugar stimulates anthocyanin metabolism and inhibits chlorophyll formation (Ji et al. 2012; Momose and Ozek 2013). Another considerable reason for the lower seed weight of purple pericarp rice might be the low chlorophyll content in the hulls and pericarps, which would reduce the photosynthetic rate and grain filling rate in the seed endosperm directly. Furthermore, the accumulation of anthocyanin in grain leads to a decline in pericarp photosynthesis, which would explain the decreased yields of black rice. During the early stages of wheat grain development, the photosynthesis rate increased with increasing level of chlorophyll content in wheat pericarp which reached maximum at 16 days after anthesis and later decreased with degradation of chlorophyll (Xiong et al. 2013). In this experiment, the progenies of crosses showed that only one allele of the *Pp* gene of the purple pericap (*Prp*) trait differed between purple pericarp rice (*Pb_Pp_*) and brown (*Pb_pppp*) or white pericarp rice (*pbpbpppp, pbpbPp_*) (Rahman et al. 2013). Therefore, the small seed size of purple pericarp rice resulting in its reduced yield potential may not be correlated with yield related genetic traits but instead with physiological factors associated with anthocyanin synthesis. Because black rice is economically important, it will be necessary to develop a large grain size for breeding. The results presented herein will improve future breeding techniques to develop high yield black rice varieties containing high amounts of anthocyanin.

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