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The *Purple leaf* (*Pl*) Alleles, Pl^w and Pl^i, Regulate Leaf Color Development Independently from the *Pb* Gene of *Purple pericarp* (*Prp*) in Rice

Sang Gu Kang [1,*], Kyung Eun Lee [2], Jegeun Cho [1], Jeong Wook Lee [1], Geum Sook Do [3] and Mohammad Nurul Matin [1,4,*]

[1] Department of Biotechnology, Institute of Biotechnology, College of Life and Applied Sciences, Yeungnam University, Gyeongsan 38541, Republic of Korea; garcurse@naver.com (J.C.); eel7777@naver.com (J.W.L.)
[2] Sunforce Inc., 208-31, Gumchang-ro, Yeungcheon-si 31882, Republic of Korea; keun126@ynu.ac.kr
[3] Department of Biology, College of Natural Sciences, Kyungpook National University, Buk-gu, Daegu 41566, Republic of Korea; dogs1004@hanmail.net
[4] Molecular Genetics Laboratory, Department of Genetic Engineering and Biotechnology, University of Rajshahi, Rajshahi 6205, Bangladesh
* Correspondence: kangsg@ynu.ac.kr (S.G.K.); nmatin@ru.ac.bd (M.N.M.)

Abstract: Color development in various rice organs results from the complementary expression of genes involved in anthocyanin biosynthesis. The *Purple pericarp* (*Prp*) trait and the *Purple leaf* (*Pl*) trait both display epistasis, relying on the complement of the *Pb* and *Pp* genes for pericarp coloration and the *Pl* and *Pp* genes for leaf coloration, respectively. However, there is still genetic uncertainty in identifying the genes responsible for the various color expressions and intensities of rice grain pericarp and leaves. In this study, we characterized the inheritance patterns of color development and the mode of anthocyanin pigments in rice by crossing two parental mutant lines. YUM051, exhibiting dark purple leaves (Pl^w) and purple pericarp (*Prp*), was crossed with YUM144, which displayed light purple leaves (Pl^i) and a white pericarp (*prp*). The F1 plants exhibited dark purple leaves with purple pericarps, indicating the dominant nature of the *purple leaf* (*Pl*) and *purple pericarp* (*Prp*) traits. The rice *Prp* traits display a complementary interaction, reflected in a 9:7 ratio of purple to white pericarp. However, the *Prp* trait followed Mendelian segregation with a 3:1 ratio of purple to white pericarp in this cross, indicating homozygous dominant *Pp* alleles in both parental plants. Meanwhile, the segregation of the purple leaf color in the F_2 generation of this cross followed complementary inheritance, exhibiting a 9:7 segregation ratio between purple leaves and greenish leaves with purple leaf margins. Moreover, the co-segregation of *Prp* and *Pl* traits in the cross between YUM051 (Pl^w) and YUM144 (Pl^i) plants did not adhere to the Mendelian 9:3:3:1 independent assortment ratio, confirming that the *Pl* gene and *Pb* gene are linked on the same chromosome. Cyanidin-3-O-glucoside (C3G) was detected in the leaves of all progeny resulting from the Pl^w and Pl^i cross. However, C3G was exclusively identified in the seeds of offspring carrying the dominant *Pb* gene. Therefore, the Pl^w and Pl^i alleles are *Pl* genes responsible for purple leaf color, while the *Pb* gene is responsible for purple pericarp color in rice; these genes function independently of each other.

Keywords: anthocyanin; *Oryza sativa*; purple pericarp; purple leaf; epistasis; complementary inheritance

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1. Introduction

In several rice (*Oryza sativa* L.) cultivars, a rarely observed phenotype of purple coloration appears on various organs, including leaf blades, leaf sheaths, palea, lemma, nodes, internodes, awns, hull, pericarp, stigma, and apiculus. This phenomenon is attributed to the accumulation of pigments, such as anthocyanins, resulting in the development of dark purple, light purple, or purple-washed leaves, as well as red, brown, black, or purple grain

pericarp [1–12]. To understand the fundamental principles of color genetics, rice geneticists have studied the mode of inheritance in rice since Mendel's experiments in the 19th century. The anthocyanin pigmentation in rice is primarily governed by the dominant genes *C* (chromogen), *A* (activator), and *P* (regulator). Both C and A are crucial for pigmentation, while P serves as a tissue-specific regulator for both C and A [11,13–16]. According to the C-A-P system, genes such as *OsC1*, *OsDFR,* and *OsB1* and *OsB*2 were individually identified as determinants of anthocyanin biosynthesis in rice, respectively [11,15,16]. Subsequently, *OsKala1* (*DFR*) and *OsKala4* (*OsB2*) have been described for colorization in rice [9,17].

The *purple leaf* (*Pl*) gene in rice comprises three alleles, *Pl*, Pl^W, and Pl^+, at the *Pl* locus of rice chromosome 4, which regulates anthocyanin pigmentation in various tissues. The distribution effects of *Pl* and Pl^W are similar, showing coloration in the leaf blade, leaf sheath, collar, auricle, ligule, node, and internode. However, the intensity of coloration may vary. For instance, coloration attributed to Pl^W tends to fade out during the maturation stage, whereas coloration attributed to *Pl* does not show any noticeable change until maturity [11,18]. The *Pl* locus contains the *Pb* and *Pl* genes responsible for anthocyanin biosynthesis, producing purple pericarp and leaves, respectively [8,11]. The *Pb* gene is located on Os04g0557800, corresponding to the *OsB1* gene encoding an R-type basic helix–loop–helix (bHLH) transcription factor (https://rapdb.dna.affrc.go.jp/; accessed on 25 December 2021) [8]. The *Pl* gene is located on Os04g0557500, corresponding to the *OsB2* gene encoding a basic helix–loop–helix (bHLH) transcription factor (https://rapdb.dna.affrc.go.jp/; accessed on 25 December 2021) [11].

Diverse grain pericarp colors, ranging from white, brown, and red to black, have been reported in rice as *Ra* and *Rc* genes [19] and have been intensively reviewed [20]. Anthocyanin pigmentation and the *purple pericarp* (*Prp*) trait in rice are genetically determined by the complementary interaction of two dominant genes, *Pb* and *Pp*, which have been identified on chromosomes 4 and 1, respectively [8,21–23]. Subsequently, they were identified as *Kala4* and *Kala1*, respectively, which determine the pericarp color of rice [9,24]. The *Pp* allele is dominant over the wild-type white pericarp *pp* allele [8,23]. The rice pericarp acquires a brown color when the *Pb* gene is present even in the absence of the *Pp* gene, whereas no coloration occurs in the absence of the *Pb* gene [8,23]. Moreover, the pericarp acquires black coloration in the presence of both dominant *Pb* and *Pp* alleles, i.e., *Pb_Pp_* results in a purple pericarp, *Pb_pppp* results in a brown pericarp, and pbpbPp_ and *pbpbpppp* result in a white pericarp [8,25,26].

The demand for colored grains with a high anthocyanin content is increasing worldwide because of their significant functional attributes, including antioxidant properties, anticancer effects, retinal protection, antihypolipidemia effects, antiaging and health improvement properties, as well as their high nutritional value [26–28]. Breeders and consumers often prefer pigmented rice cultivars because they have commercial value, attributed to the antioxidant and health-promoting properties of pericarp anthocyanins [29,30]. Moreover, high levels of anthocyanins, amino acids, and free fatty acids in purple rice confer health advantages, such as protection and prevention against angiogenesis and cardiovascular diseases [31–33]. Leaf color mutants may be an important morphological marker in hybridization [34–36], which also provides defense against biotic and abiotic stresses [15,37], and they are a good source for studying pigment metabolism, chloroplast development, and differentiation [29–33,38], as well as for candidate gene identification [39]. Therefore, studying rice colorization has become a crucial objective in rice genetics and breeding to enhance agronomic traits [9,19,28,29].

Colorization in cereals is caused by the accumulation of carotenoids and flavonoids, such as anthocyanin, proanthocyanin, and phlobaphene, in the pericarp, aleurone, lemma, testa, and seed coat of grains and leaves [26]. Rice leaves can become purple, brown, or red because of the accumulation of various flavonoids owing to specific gene expression in various genotypes. Cyanidin-3-glucoside (C3G) and peonidin-3-glucoside are the two main pigments deposited in the pericarps of purple rice [7,8]. Therefore, we examined the C3G content in the leaves and seed pericarps of the colored leaf parents.

Inheritance studies on coloration commenced early in the field of rice genetics, and genes associated with color development in cereals have been extensively studied [1,26]. However, because the expression and distribution of anthocyanin pigmentation and concentrations of various colors in rice organs are strikingly variable, the determination of the genes involved in color development remains undetermined, and only a few functional and allele-specific markers for classical breeding have been reported thus far. Several studies have examined crosses between pigmented and non-pigmented leaf blades; however, very few have examined crosses between two pigmented leaf blade types. Therefore, in this study, we defined the inheritance patterns of associated genes among progenies derived from crosses between two mutant lines of *Pl* alleles exhibiting distinct color phenotypes in the leaves and pericarps.

2. Materials and Methods

2.1. Plant Materials

Two purple leaf rice lines, YUM051 (Pl^w) and YUM144 (Pl^i), were cross-fertilized in a reciprocal manner and grown in the greenhouse and rice research field at Yeungnam University, Gyeongsan, Republic of Korea. The genotype YUM051 (Pl^w) had dark purple leaves, purple seed pericarps, internodes, and leaf collars, whereas YUM144 (Pl^i) had light purple leaves, white pericarps, and white leaf collars. Recombinant inbred lines of the crossbred progeny were obtained from subsequent generations.

2.2. Genetic and Phenotypic Characterization

A genetic study was conducted to analyze the inheritance patterns of color formation in various organs. The pigmentation of various organs in the experimental materials was documented, including the leaf sheath, leaf blade, leaf margin, stem, node, internode, auricle, ligule, collar, hull, and pericarp. The purple-colored genotypes YUM051 and YUM144 were hybridized, and the resulting F_1 plants were allowed to self-fertilize to produce F_2 and subsequent generations. The phenotypic data were visually assessed for 147 and 213 plants from the F_2 generation of the YUM051(♀) X YUM144(♂) and YUM051(♂) X YUM144(♀) crosses, respectively. The segregation patterns of color development in specific organs were individually documented. The χ^2 method was applied to validate the results. For genetic analysis, genomic DNA was extracted from young leaf tissues using the CTAB method, following a standard protocol [40]. Allelic differences in the *Prp* trait among the progenies were investigated using single-nucleotide polymorphisms of the *Pb* gene encoding *OsB1* mRNA (NM_001060067) in the genome sequences of the *O. sativa* Japonica group (Os04g0557800). The allelic constitutions of the *Pb* gene were determined through the analysis of polymorphisms within the *OsB1* DNA sequence (AB021079) via polymerase chain reaction (PCR) [8]. Briefly, PCR, for amplifying the transcriptional activator *Pb* gene, was performed using a forward primer 5′-GGGAGAAGCTCAACGAGATG and a reverse primer 5′-GGGTGGCAGATTCATCACTT. For genotype analysis, PCR products were digested with BamH1 restriction enzyme (Promega Co., Madison, WI, USA), followed by gel electrophoresis on a 1.2% agarose gel.

2.3. High-Performance Liquid Chromatography (HPLC) Analysis of Anthocyanin Contents

Because the purple coloration of rice is caused by the accumulation of anthocyanins [7,8,41], we analyzed the anthocyanin content in the leaves and grains. Anthocyanin extraction and content analysis were performed following a previously described protocol with modifications [42,43]. Briefly, 10 g of rice leaf powder and 10 g of seed pericarp powder (obtained by milling brown rice with an automatic rice tester) were extracted individually using 50 mL of 70% ethanol as the extraction solvent for 24 h at 25 °C in the dark. Subsequently, the solution was centrifuged at 10,000× *g* for 20 min and filtered through a 0.25 µm PVDF filter (Millipore, Billerica, MA, USA). Ten microliters of the filtered samples were injected into an HPLC system (Sheseido, Tokyo, Japan). 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 comprised 5% formic acid (solvent A) and 5% acetonitrile containing 5% formic acid (solvent B). Elution was conducted using a linear gradient of B to A at a flow rate of 1.0 mL/min as follows: the elution began with 0–35.5% B at 0–23 min under isocratic flow, and increased from 35.5 to 100% B at 24–45 min. Kuromanin (Sigma, St. Louis, MO, USA) was used as a reference standard chemical for the measurement of C3G.

3. Results

3.1. Phenotypic Differences between Pl^w and Pl^i Mutants

In this study, we analyzed rice genotypes exhibiting color development in various organs. Pl^w and Pl^i are the two alleles of *Pl*-type purple-leaved rice that we studied intensively in the two parent lines. YUM051 had uniformly dark purple coloration in the leaf blade (Pl^w) with a deep purple auricle (*Pau*), internode, purple pericarp (*Prp*), and purple hull (*Pr*). In contrast, YUM144 showed a less intense purple coloration in the leaf blade (Pl^i) and stems, with a white pericarp (*prp*) and hull (*pr*) (Figure 1 and Table 1). Both the Pl^w and Pl^i parents were reciprocally hybridized to analyze color segregation in specific tissues. The coloration was segregated with various patterns in the different organs of the subsequent progenies. Because of the many patterns of color distribution, it was difficult to characterize the progenies in the F_2 generation. Therefore, individual progenies showing a unique phenotype from self-pollinated populations were selected. Consequently, we analyzed seven generations of offspring with fixed alleles for the *Pl* and *Prp* traits of interest (Figure 1).

Table 1. Pattern of color development in the two purple leaf-type parents and their progenies.

Genotype. ID	Type	Leaf					Stem		Spikelet	
		Blade	Sheath	Auricle	Collar	Ligule	Node	Internode	Pericarp	Hull
YUM051	Pl^w	++	++	++	-	+	-	++	++	+
YUM144	Pl^i	+	+	+	-	+	-	+	-	-
F_7-03	Pl^i	+	+	+	-	+	-	+	-	-
F_7-07	Rec	+	+	+	-	+	-	+	+	+
F_7-08	Rec	-	-	-	-	-	-	-	++	+
F_7-13	Pl^w	++	++	++	-	+	-	+	++	+
YUC044	pl^+	-	-	-	-	-	-	-	-	-

++: dark purple, +: light purple, -: no color/green, F_7 is progenies from 7th generation of the cross between Pl^w and *Pli* parents. Rec: recombinant type.

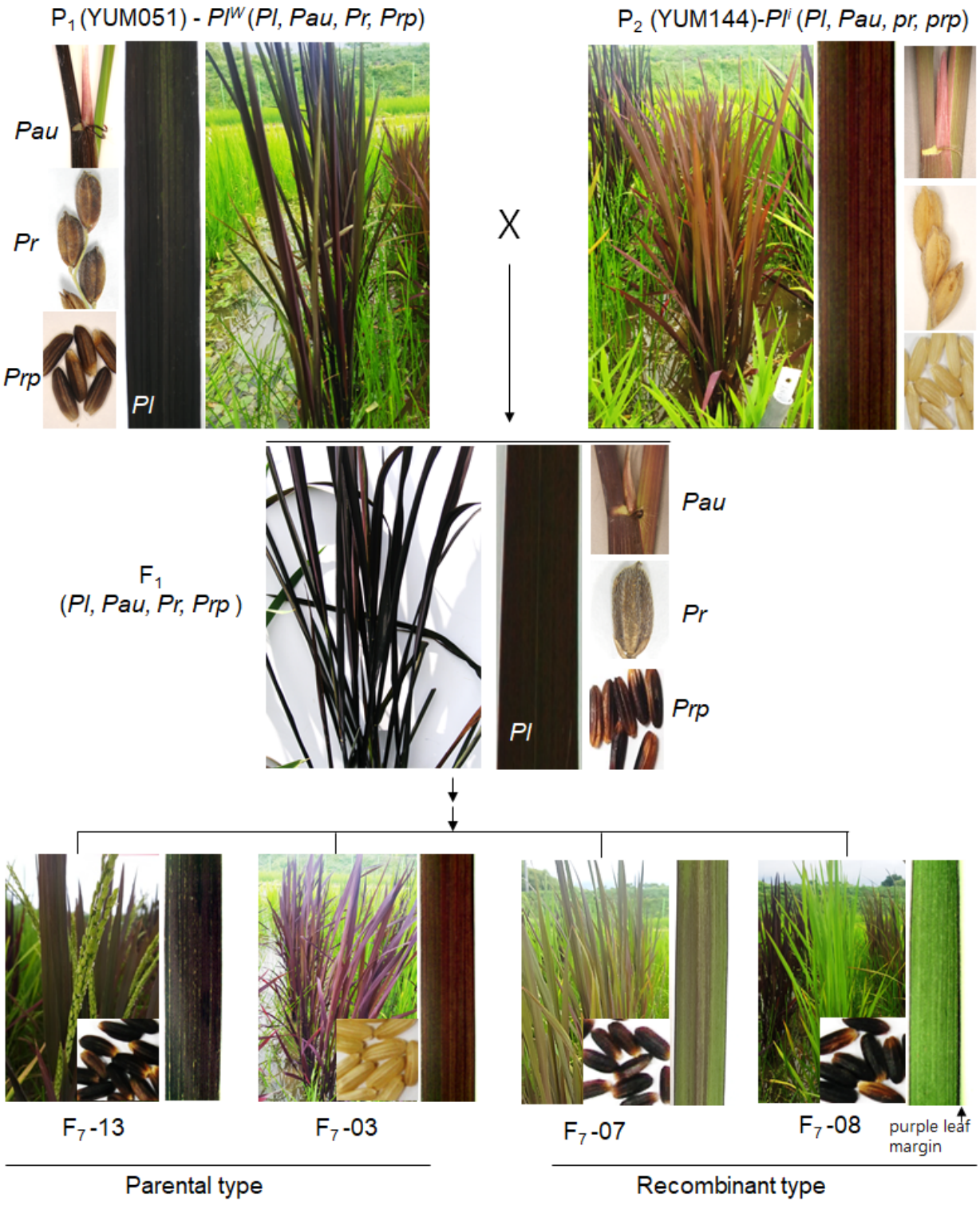

Figure 1. Phenotypes, gene segregation patterns, and allelic analysis of purple leaf parents and their recombinant inbred lines in the F_7 generation used in this study. The pigmentation in various tissues of the materials is indicated. The P_1 parent (YUM051) exhibiting dark purple leaf (Pl^w), purple auricle (*Pau*), purple hull (*Pr*), and purple pericarp (*Prp*), was hybridized with the P_2 parent (YUM144) exhibiting light purple leaf (Pl^i), with white pericarp and no color on the hull. F_1 showed a phenotype similar to that of the P_1 parent. *Pl*: Purple leaf, *Prp*: purple pericarp, *Pau*: purple auricle, *Pr*: purple hull, *prp*: white pericarp, *pr*: wild-type hull.

3.2. Differential Color Segregation on Progenies Crossed between Pl^w and Pl^i Allele Parents

Purple color segregation from two purple-colored parents was studied in a cross between YUM051 and YUM144. The color of the F_1 plants generated from the hybridized seeds displayed purple phenotypes in the leaf blade, leaf sheath, auricle, ligule, collar, and internode, as well as on the hull and pericarp of the seeds developed in the F_1 plant (Figure 1). Similar results were obtained from reciprocal crosses whenever Pl^w, as a female parent pollen receiver, was crossed with Pl^i, as a male parent pollen donor, or vice versa. This pattern of coloration indicates that the purple color traits in these crosses adhered to the dominant genetic characteristics.

The F_1 plants were allowed to self-fertilize to produce the F_2 segregating generation, and the development of various colors within them was analyzed. Among the 213 progenies from the F_2 population, 114 plants had purple leaves and 99 plants had greenish leaves. The greenish leaves were light green specified with purple leaf margins. The absence of completely green leaves in this cross resembles the allelic relationship between Pl^w and Pl^i. The Chi-square (χ^2) tests did not follow any significant ratio in the genetic pattern of segregation ($\chi^2_{3:1}$ = 52.40, P = NS, Table 2). The reciprocal cross also showed similar segregation patterns. However, the calculated χ^2 fitness to the segregation ratio of leaf color, 9:7 ($\chi^2_{9:7}$ = 0.64, P = 0.90–0.10), for the cross between YUM144 and YUM051 supports the complementary inheritance of Pl^w and Pl^i (Table 2).

Table 2. Segregation of the purple leaf color in the F_2 generation from the reciprocal crosses between the YUM051 and YUM144 parents.

Cross ID	Cross (Pollen Donor X Pollen Recipient)	F_1 Phenotype		Segregation in F_2			χ^2	*p*-Value
				Purple	Greenish/plm	Total		
SGK07086	YUM144 × YUM051	Purple leaf	Obs.	114	99	213	χ^2 (3:1) = 52.40	NS
			Exp.	159.75	53.25			
			Obs.	114	99	213	χ^2 (9:7) = 0.64	0.90–0.10
			Exp.	119.8	93.18			

SGK07086: lab cross ID, Obs.: Observed number, Exp.: expected number, χ^2: Chi-square, *p*: probability value, Greenish/plm: greenish leaves with purple leaf margins.

Conversely, in the context of the purple pericarp color, the F_1 plants resulting from a cross between plants with purple and white pericarp exhibited a dark purple pericarp that separated in the F_2 generation at a 3:1 (purple:white) ratio in reciprocal crosses ($\chi^2_{3:1}$ = 1.41 and 2.89, respectively, P = 0.90–0.10) (Table 3). This segregation ratio indicates the dominant nature of the *Pb* gene controlling the *Prp* trait. Moreover, the segregation analysis indicated that the *Pl* and *Pb* genes are linked in inheritance, as they did not follow the Mendelian 9:3:3:1 independent assortment ratio in the F_2 generation [$\chi^2_{(9:3:3:1)}$ = 117.41, P = NS] (Table 4). Furthermore, four different groups of color patterns were distinguished in subsequent generations, constituting both parental and recombinant types. The parental type comprised plants with dark purple leaves bearing purple pericarps and light purple leaves bearing white pericarps. The recombinant type included plants with purple pericarps exhibiting either faintly purple or green leaves, with a purple leaf margin (Figure 1 and Table 4). In the F_7 generation, the presence of a few recombinant types of plants demonstrated that the parental genes were linked to each other, that is, purple leaf-and purple pericarp-controlling genes (*Pl* and *Pb*) were linked in inheritance.

Table 3. Segregation of the purple pericarp color in the F_2 generation from the crosses between the YUM051 and YUM144 parents.

Cross ID	Cross Combination	F_1 Pericarp Phenotype		Segregation in F_2			χ^2 (3:1)	*p*-Value
				Purple	White	Total		
SGK07085	YUM051 × YUM144	Purple	Obs.	104	43	147	1.41	0.90–0.10
			Exp.	110.25	36.75			
SGK07086	YUM144 × YUM051	Purple	Obs.	149	64	213	2.89	0.90–0.10
			Exp.	159.75	53.25			

SGK07085 and SGK07086: lab cross ID, χ^2: Chi-square, *p*: probability value.

Table 4. Co-segregation of the *Pl* and *Prp* traits at the F_2 generations from the dihybrid cross.

ID	Cross Combination	F_1 Phenotype		F_2 Segregation					χ^2 (9:3:3:1)	*p*-Value
				Pl, Prp	*Pl, prp*	*pl, Prp*	*pl, prp*	Total		
SGK07086	YUM144 × YUM051	Dark purple leaf, purple pericarp	obs.	99	15	50	49	213	117.41	NS
	(+, +) × (*Pl*, *Prp*)		exp.	119.83	39.93	39.93	13.31	213		

SGK07085: lab cross ID, +, +: considering wild type normal for both characters, *Pl*: dominant for dark purple leaf color, *pl*: other leaf colors, *Prp*: dominant for purple pericarp color, *prp*: white pericarp color. obs., observed number: exp, expected number: NS, non-significant.

3.3. Anthocyanin Contents Were Related to Color Development in the Rice Organs

We analyzed the anthocyanin content in the leaf and seed pericarp. Leaf extracts from the green leaf control plant (Ilpoom), the dark purple (Pl^w), and diluted purple (Pl^i) parents, along with their two F_7 progenies exhibiting purple and green leaf with purple leaf margins, were subjected to HPLC analysis. A prominent peak for C3G was observed in the Pl^w, Pl^i, and F_7-03 offspring with dark purple leaves. In contrast, a small amount of kuromanin was detected in the F_7-08 offspring, with green and purple leaf margins (Figure 2A). The presence of this negligible amount of C3G in F_7-08 may have contributed to the pigmentation at the leaf margin (Figure 1). Control plants with green leaves showed no pigment signals (Figure 2A).

In another experiment, the anthocyanin content of the seed extracts from the parents and their progenies was measured. The Pl^w parent and F_7-08 offspring with purple seed pericarps and one reference plant with black seed pericarps had significant amounts of C3G, as they produced relative signals, whereas the Pl^i parent and F_7-03 offspring with white pericarps exhibited no C3G in their extracts, as no detectable peak was observed (Figure 2B).

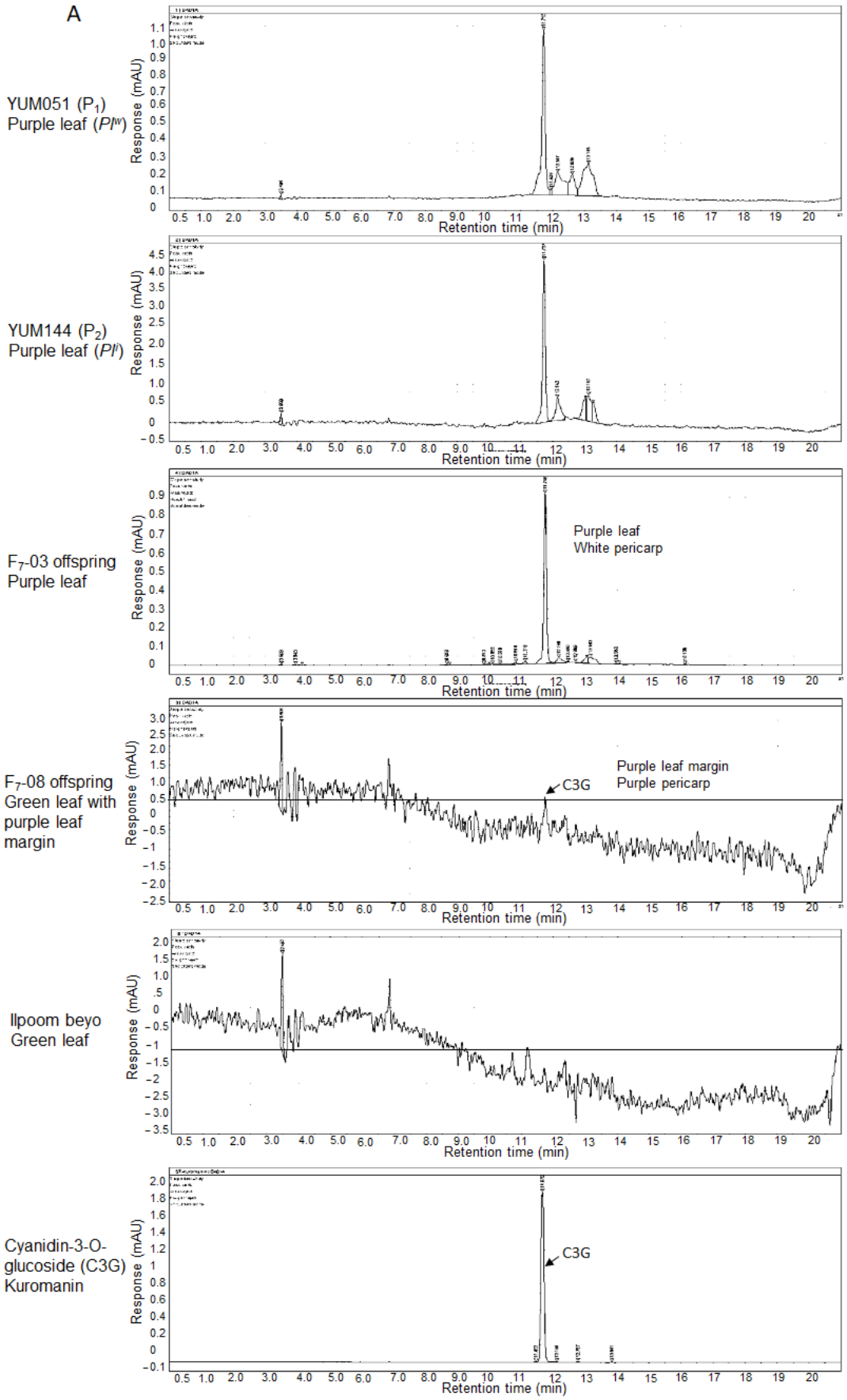

Figure 2. *Cont*.

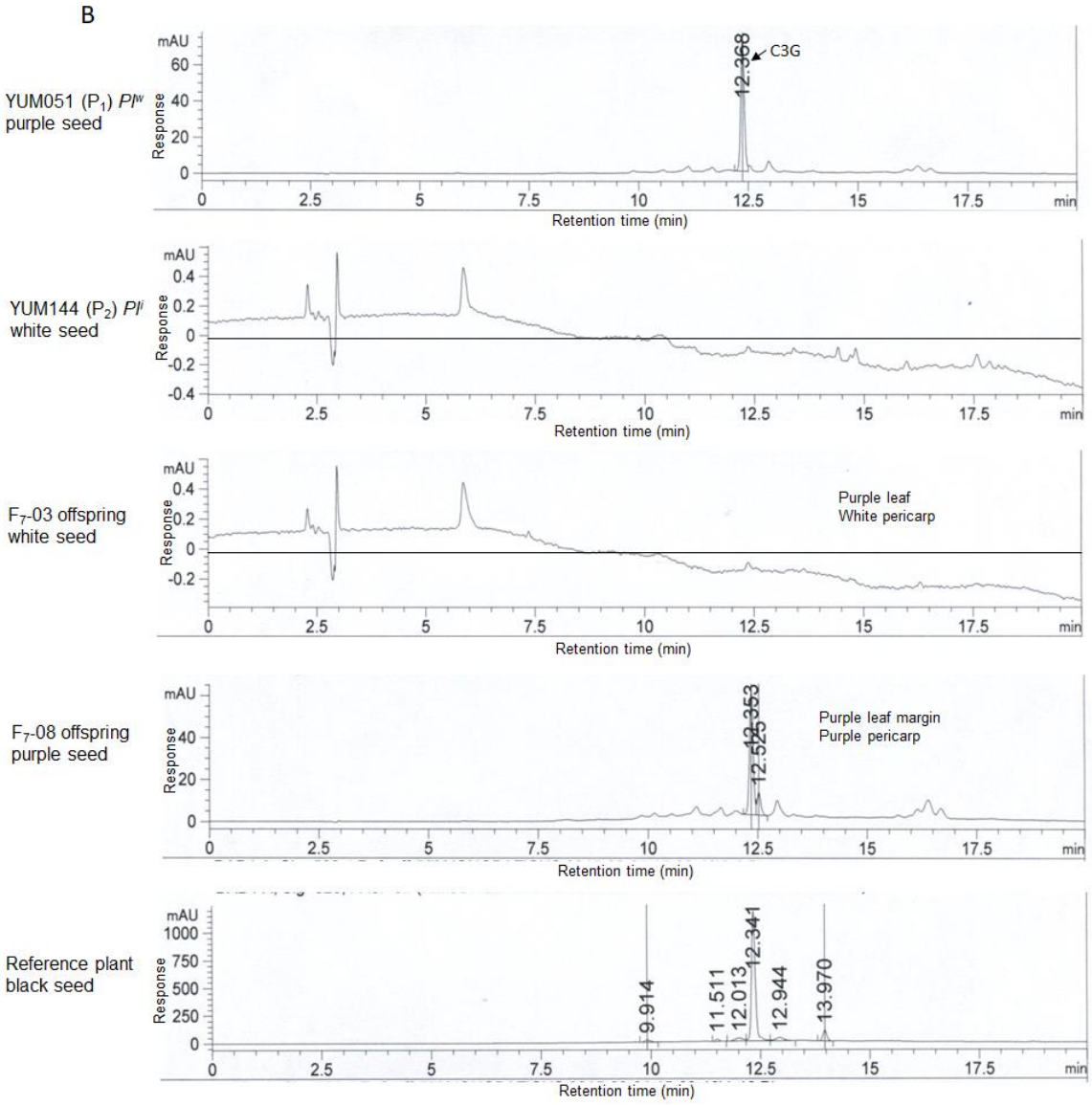

Figure 2. Anthocyanin profiles of the leaf and pericarp extract using high-performance liquid chromatography. (**A**) Anthocyanin profiles of the control, parents, and progenies of recombinant inbred lines of purple leaf and green leaf extracts analyzed using high-performance liquid chromatography. The peak is C3G (kuromanin) in standard. The retention time is indicated on the horizontal line, and the amount of absorption unit (mAU) is indicated on the vertical line. High amounts of C3G were detected in both parents and progeny (F_7-03) with purple leaf. However, a negligible amount of C3G was detected in the progeny (F_7-08) having green leaf with purple leaf margin. C3G was not detected in the control plant with green leaf. (**B**) The anthocyanin content of the seed extract of the parents and their progenies. Pl^w parent and offspring (F_7-08) with purple seed, as well as the control with black seed contained high amounts of C3G, whereas the Pl^i parent and offspring (F_7-03) with white seed did not contain C3G in their extracts. Retention time is indicated on the horizontal line, and the amount of absorption unit (mAU) is indicated on the vertical line.

3.4. Genetics of Pigment Segregation and Differential Expression of Pl and Pb Genes

We analyzed the genetic patterns of pigment segregation and development in various tissues between the parents and offspring in a cross between YUM051 and YUM144. Figure 3 demonstrates that the cultivar with purple pericarp seeds (YUC020) had purple color in all vegetative parts, including the leaf, leaf sheath, internode, ligule, auricle, and reproductive parts of the seed pericarp. The other cultivar with uncolored pericarp seeds

(YUC044) did not show color development in any organ (Figure 3A). Color development was observed in the recombinant inbred lines (Figure 3A). In the offspring SGK07085-142-08-02-06-16, the leaves and leaf sheaths were green, but the internodes, ligules, auricles, and pericarps were purple, resembling the coloration of the recombinant type. In contrast, in the SGK07085-142-10-01-04-01 offspring, the leaf and leaf sheath were purple, but the internode, ligule, and pericarp were not, and they also appeared to be of the recombinant type. The SGK07085-142-10-01-10-02 offspring were similar to P_1, but the leaf color was not as dark as that of P_1. The SGK07085-142-08-02-01-01 F_5-04 offspring also displayed a phenotype similar to that of P_1, but it was still not completely pigmented in the internode. The other two offspring, SGK07085-142-08-02-01-07 and SGK07085-142-10-04-04-01, exhibited coloration similar to that of P_1, but the leaf color differed in SGK07085-142-08-02-01-07, and a white pericarp was observed in SGK07085-142-10-04-04-01 (Figure 3A). The diverse and complex patterns of color segregation indicate that these genes may individually regulate color formation in different organs.

We also analyzed the composition of the *Pb* gene, which encodes the *OsB1* transcription factor in the *Prp* trait [8,11]. The purple leaf trait is determined by the *Pl* gene, which encodes the OSB2 protein. The allelic constitutions of the inbred lines were analyzed through PCR-based polymorphism of the *OsB1* DNA sequences (*Pb* gene). The *OsB1* PCR products (*Pb*) were digested with BamH1 restriction enzyme. The dominant *Pb* allele contained 5′-GGATCC sequences in OsB1, which were cut by BamH1. The recessive *pb* allele was a 2-bp (GT) insertion in the sequence of the *OsB1* DNA sequences, which resulted in a 5´-GGATGTCC sequence that was not cut by BamH1. The 1.2-kb fragments of the *OsB1* (*Pb*) gene were successively differentially produced in the progeny (Figure 3). The recessive mutated alleles *pb* amplified the single band in the white pericarp parent (YUM144), white pericarp control (YUC044), and progenies in the F_5 generation with white pericarp (SGK07085-142-10-01-04-01 and SGK07085-142-10-04-04-01), as they were not cut by BamH1 (Figure 3B). The dominant normal gene *Pb* was identified as two fragments in the parents (YUM051), control (YUC020), and progenies in the F_5 generation having purple pericarps as they were cut by BamH1 (Figure 3B). Nonetheless, one offspring (SGK07085-142-10-01-10-02) with a purple pericarp produced three DNA bands, possibly because of heterozygosity.

Here, we demonstrated that the *Pb* and *Pl* genes are independent of genetic functions and are involved in the pigmentation of purple pericarps and leaves in rice, respectively. Moreover, *Pl* traits are controlled by epistatic gene interactions with *Pl*, Pl^{w}, and *pl* with *Pp* genes, leading to the purple coloration of the leaves.

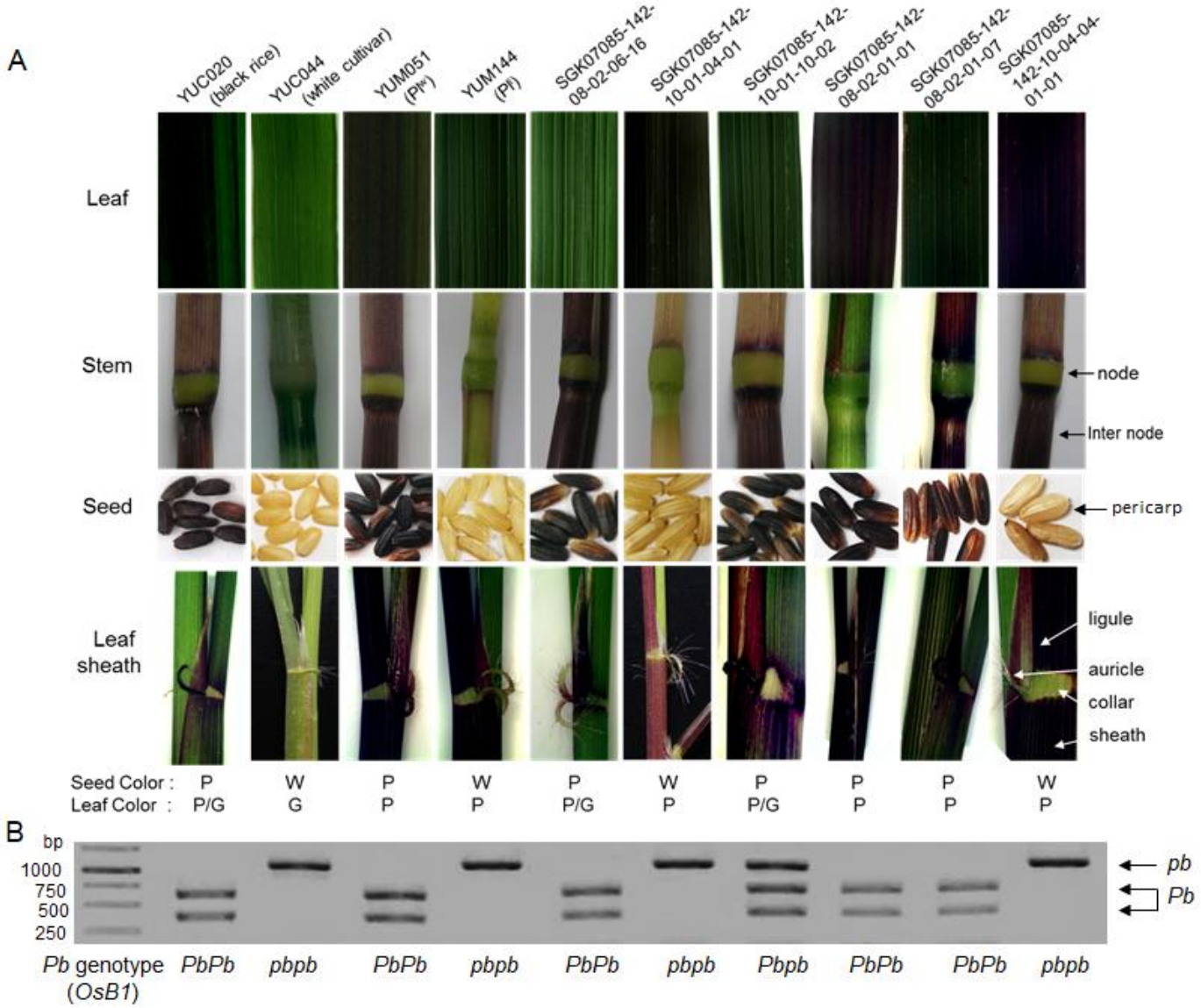

Figure 3. Genetics of phenotypic variation of pigment formation across different rice tissues and restriction enzyme analysis of the purple-pericarp-specific gene *Pb* (*OsB1*) [8]. (**A**) Segregation of color development at various tissues in the control, parents, and recombinant inbred lines. Color development at the leaf, stem, seed, leaf sheath, ligule, auricle, and collar is demonstrated. Each panel row corresponds to its ID. (**B**) Tissue-specific single nuclear polymorphism analysis of the *Pb* (*OsB1* DNA) gene in genotypic and phenotypic constitutions in the corresponding plants in panel A. PCR-amplified genomic DNA of *OsB1* were cut with restriction enzyme BamHI. The genotypes were as follows: *PbPb* for the homozygous dominant allele exhibited two bands. *Pbpb* for the heterozygous allele exhibited three bands. *pbpb* for the homozygous recessive allele exhibited one band. Phenotypes are indicated by the pericarp colors, with P for purple and W for white; leaf color is denoted as follows: P for purple and P/G for green leaf with purple margin and G for green leaf. First lane: 1 kb DNA marker.

4. Discussion

Anthocyanin is the pigment responsible for the purple color of grains, leaves, flowers, vegetables, and fruits [8,11,12,44,45]. Within rice, the various colors displayed by the organs are due to the accumulation of anthocyanins, which are traits associated with domestication. The *pl* locus, which contains two adjacent genes, is responsible for leaf coloration in rice [11]. Extensive genetic analysis has revealed that chromogen *C*, activator

A, and regulator *P* are the key elements for tissue-specific coloration across various organs, with the intensity of coloration contingent upon various combinations of alleles from the *C*, *A*, and *P* loci [1,13,46]. However, a systematic understanding of the coloration of specific organs remains unclear. In this study, we studied the genetic relationship between Pl^w and Pl^i through crosses between two individually distinct colored parents and found that the leaf color of the Pl^w and Pl^i alleles showed complementary effects for their inheritance on further segregation, as they followed a Mendelian 9:7 complementary ratio (Table 2). The pattern of coloration of F_1 indicated that dark purple color traits were dominant over light purple; however, progeny segregation did not follow the Mendelian 3:1 ratio for their inheritance. In contrast, the purple leaf trait *plr4I* is regulated by a recessive gene on chromosome 4 in rice [39]. Previously, three *Pl* alleles, Pl^j, Pl^W, and Pl^i, were identified with distinct pigmentation patterns in rice leaves upon the coexistence of the high-ranked alleles *A* and *C*. Thus, the allelic strength of purple leaf genes was reported as $Pl > Pl^w > Pl^i > Pl^+$ [5]. The Pl^w allele activates dark pigmentation in the leaves, hull, and pericarp, excluding stem nodes, and Pl^j and Pl^i alleles exhibit pigmentation in the leaves, but not in the pericarp [11,18]. Nagao et al. [18] found that *Pl* and Pl^w are responsible for the development of numerous colors in leaf blades and sheaths upon coexistence with the *A* and *C* genes. Furthermore, the cross between Pl^w and Pl^i alleles was segregated following the Mendelian ratio Pl^wPl^w:Pl^wPl^i:Pl^iPl^i=1:2:1 in the F_3 generation [3,5]. The colored leaf sheath in rice *PSH1* was identified as the dominant complementary interaction between *OsC1*, and *Rb1* and *Rb2* control the purple leaf sheath [47]. A genetic analysis of the deep purple and light purple parents produced dark purple, light purple, and green offspring in the F_2 segregating generation in a 39:12:13 ratio, suggesting that trigenic differences between purples suppress one of the genes' function [4].

We also demonstrated the involvement of *Pb* and *Pl* in grain pericarp pigmentation. Individually, *Pb* produced purple grains, whereas *Pl* produced purple leaves. The rice purple pericarp (*Prp*) trait is controlled by complementary gene interactions between the dominant alleles of *Pb* and *Pp* genes to produce colored rice seeds [23]. The F_1 plants, derived from a cross between purple and white grain pericarps, exhibited grains with dark purple pericarps. Moreover, the offspring segregated at a 3:1 ratio in the F_2 generation (Table 3), indicating the dominant nature of the *Pb* gene controlling purple grain color. Furthermore, we found from the segregation analysis that the *Pl* and *Pb* genes are linked in inheritance.

The purple color of rice is attributed to the high level of accumulation of pigments such as anthocyanins, lending the leaves and seed pericarps a dark purple color [1,7]. Therefore, we measured the anthocyanin C3G content in the pigmented portion of rice as an important factor in determining the phenotype (Figure 2). These results are in accordance with the coloration of the materials. Moreover, the negligible amount of C3G in the green progeny may be owing to the pigmentation at the leaf margin. These results indicated the involvement of anthocyanins in color formation in rice. The accumulation of anthocyanins, such as C3G and peonidin 3-O-glucoside, determines the color of the leaf sheath (*PSH1*) in rice, which was identified as *Rb1* and *Rb2* [47]. However, previous genetic studies on the *Pl* trait may have provided insufficient phenotypic data for analysis by considering green leaves despite the presence of negligible amounts of anthocyanins. This assumption can be attributed to the limitations in the technological advancement of HPLC machines and DNA sequencing in previous decades. Although they could not accurately measure the depth of the purple coloration in rice, a genetic analysis of the complex *Pl* trait showed remarkable agreement with epistasis. Additionally, we previously demonstrated that a complementary genetic relationship of epistasis contributes to the depth of purple coloration of the seed pericarp color in rice [8].

Plant materials with colored leaves and pericarps are of potential interest because of their important nutritional functions and could serve as a good source of phenotypic markers in rice breeding [48,49]. In this study, using two distinctly individually colored mutant rice lines, we confirmed the initial hypotheses as follows: the *Pb* gene for OsB1 is

responsible for determining the seed pericarp color (*Prp*) trait, but not the leaf color (*Pl*) trait in the Pl^w and Pl^i alleles. The Pl^w allele was found to be more dominant than the Pl^i allele. Therefore, understanding these genetic factors is crucial for understanding the purple coloration of rice leaves and pericarps.

5. Conclusions

In this study, we demonstrated that the *Pl* gene enables the accumulation of anthocyanin pigments in rice leaves, where the Pl^w allele produces dark purple leaves, while the pl^i allele regulates light purple leaves in rice. Genetic identification revealed that the Pl^w allele is dominant over pl^i allele. The *Pl* gene is independent of the *Pb* gene, based on a complementary analysis of crosses between Pl^w and pl^i allele plants.

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