COMPARATIVE EXPRESSION OF ANTHOCYANIN-RELATED GENES, PIGMENT ACCUMULATION, AND GRAIN QUALITY TRAITS IN A BLACK-KERNEL MUTANT AND WILD-TYPE INDICA RICE (*Oryza sativa L.*)

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Abstract
Pigmented rice is a rich dietary source of anthocyanins and flavonoids with potential health benefits, with limited yield and quality trade-offs. However, the relationship between pigment accumulation, agronomic performance, and grain quality remains poorly defined. In this study, we conducted a comparative analysis of a gamma-ray-induced black-kernel rice mutant (9311 bk) and its wild-type progenitor (9311). Agronomic, physiological, and biochemical traits were evaluated under field conditions. The mutant exhibited taller plants and longer grains, but had a reduced seed-setting rate and 1000-grain weight. Quality assessment revealed significantly greater grain length and width but reduced brown rice recovery rate and amylose content in the mutant compared to the wildtype. Biochemical assays revealed significantly higher anthocyanidin, flavonoid, and polyphenol accumulation in 9311 bk grains, coinciding with strong upregulation of seven key anthocyanin biosynthetic genes (PAL, CHS, CHI, F3H, F3'H, DFR, ANS). Thermal profiling indicated an altered starch structure in the mutant. At the same time, correlation analyses showed strong positive relationships between anthocyanidin levels and gene expression, but negative correlations with starch, sucrose, and grain weight. Our findings demonstrate that while pigment-rich rice confers enhanced nutritional and functional food benefits, its induction through mutagenesis may introduce trade-offs affecting grain yield and processing quality. These insights highlight the need for balanced breeding strategies that integrate pigment biosynthesis with yield stability and grain quality traits. Practically, this study provides a framework for developing improved pigmented rice cultivars with optimized anthocyanin content, better nutritional value, and acceptable agronomic performance suitable for both health-oriented and commercial rice production systems.
Keywords: *black rice, anthocyanins, grain quality, transcriptional regulation, carbon partitioning,*

Introduction

Rice (*Oryza sativa* L.) is the primary staple food for more than half of the world's population, contributing 20–30% of daily caloric intake (FAO, 2022). Conventional white rice serves as a major energy source but contains low levels of health-promoting phytochemicals. In contrast, pigmented rice varieties such as red, purple, and black rice are rich in anthocyanins, flavonoids, and polyphenols that contribute to their strong antioxidant capacity and have been associated with reduced risks of cardiovascular disease, diabetes, obesity, and certain cancers (Shao et al., 2018; Zaidi et al., 2019; Zhang et al., 2023).

Anthocyanins in rice are synthesized through the flavonoid biosynthetic pathway, which involves a series of structural genes including *phenylalanine ammonia-lyase* (*PAL*), *chalcone synthase* (*CHS*), *chalcone isomerase* (*CHI*), *flavanone 3β-hydroxylase* (*F3H*), *flavonoid 3'-hydroxylase* (*F3'H*), *dihydroflavonol 4-reductase* (*DFR*), and *anthocyanidin synthase* (*ANS*) (Tanaka et al., 2010; Chen et al., 2020). The coordinated expression of these genes is typically regulated by MYB–bHLH–WD40 transcription factor complexes, which

fine-tune pigment accumulation in response to developmental cues and environmental factors (Qiu et al., 2019; Kim et al., 2022).

Despite these well-established biochemical pathways, the incorporation of anthocyanin pigmentation into high-yielding rice backgrounds remains a major breeding challenge. Several studies have reported that pigmented rice lines tend to exhibit reduced grain yield, lower seed-set percentage, and inferior milling recovery compared to non-pigmented cultivars (Sun et al., 2018; Shao et al., 2020). These limitations are thought to arise from metabolic competition between primary (starch) and secondary (phenylpropanoid) metabolism, as the carbon flux diverted toward anthocyanin biosynthesis may compromise carbohydrate storage during grain filling (Wang et al., 2021; Li et al., 2023). However, the extent and physiological basis of these trade-offs remain poorly understood.

Furthermore, while prior studies have profiled anthocyanin composition and antioxidant activity in pigmented rice grains, few have systematically linked pigment biosynthesis with agronomic performance, grain quality, and starch structure within the same genetic background. Most available data are derived from diverse landraces or breeding lines that differ widely in their genetic and physiological backgrounds, thereby confounding interpretations of pigment yield relationships (Kong et al., 2022). In addition, the molecular regulation of anthocyanin biosynthesis in mutagen-induced pigmented rice mutants remains largely unexplored, especially regarding how gene expression correlates with carbohydrate allocation and thermal (gelatinization) properties of starch.

To address these knowledge gaps, this study utilized a gamma-ray-induced black-kernel mutant derived from the elite *indica* cultivar 9311 (designated 9311 bk) and its wild-type progenitor (9311) to dissect the physiological, biochemical, and molecular mechanisms associated with pigment accumulation. We assessed differences in agronomic traits, grain quality, anthocyanin and flavonoid levels, thermal properties, and transcriptional regulation of key biosynthetic genes. By integrating these datasets, we aimed to elucidate the mechanistic basis of pigment yield trade-offs and provide insights for the rational development of nutritionally enhanced pigmented rice cultivars that maintain desirable agronomic and processing quality.

Materials and Methods

Plant Materials

Two indica rice genotypes, 9311 and its corresponding mutant with the black kernel phenotype, referred to as 9311 *bk* were used in the study. The wild type 9311 was a well-known commercial indica rice cultivar with its full genome sequence accessible (http://rise2.genomics.org.cn/). The *bk* mutant was derived from 9311 cultivar (*Oryza sativa* L.ssp. indica) by gamma-irradiating 9311 mature seeds, and the stably inherited mutant was acquired through the sequential self-pollination and profiling of plant phenotype in M2 generations, with M8 seeds being exploited in this experiment. The field experiments were performed during the rainy season at the experimental station of Zhejiang University (Hangzhou, 30.16N, 120.12E), China. The field plots were randomly designed with three replications for each genotype, in which each replication was planted in 10 × 12 rows, and plant spacing was 18 cm × 18 cm with one rice seedling for each hill. The field trial was managed according to conventional practices for rice cultivation, in periodically waterlogged paddy soil, split fertilizer doses with 1.69 g kg^{-1} total N, 24.5 mg kg^{-1} available P, and 103.7 mg kg^{-1} exchangeable K.

The rice plants were sampled at grain filling stage. 60–70 panicles with uniform anthesis day were selected randomly and tagged at the full heading day. The tagged panicles were sampled from 7 to 28 days after anthesis (d) at 7 days interval. All samples were prepared in two portions; the fresh grain samples were straight away frozen in liquid nitrogen

and stored at −80◦C until further experimental analysis and the other samples were fixed in an oven at 105˚C for 30 minutes and then dried to constant weight at 80˚C. The dried samples were crushed into powder and then used for measuring soluble sugars, sucrose and starch. At maturity, 10 tagged panicles were taken from each replication and number of filled grains per panicle, seed setting rate, and grain weight were determined.

Grain qualitative traits

Various quality-related parameters like grain length (GL), width (GW), grain length and width ratio (GLWR), brown rice rate (BRR), Head milled rice rate (HMRR), chalkiness grain rate (CGR), chalkiness degree (CD) of both genotypes were evaluated. To quantify these traits, rice kernel samples were dehulled by a laboratory dehuller (Model JNMJ3, Taizhou Foodstuff Machine Factory, China) and weighed to find out the milling degree, then dehusked in a laboratory polisher to calculate milling recovery. The head and broken rice were separated through a grain separator. Hundred grains were then separated and weighed to determine GL and GW. This data was then used to calculate BRR. Dehulled rice was further milled with a miller (Model JGJ45; Qianjiang machine factory, Hangzhou, China) to obtain milled rice, which was further used to determine HMRR, CGR, and CD.

RNA Isolation, cDNA Preparation, and Real-Time Fluorescence Quantitative PCR

The procedures of RNA extraction and cDNA preparation for rice grains were performed as described previously in Zaidi et al. (2019). Trizol Plus reagent kit (Invitrogen, Carlsbad, CA, USA) was used for the extraction of total RNA, and the First Strand cDNA Synthesis kit (Toyobo, Osaka, Japan) was used for cDNA synthesis by following the manufacturer's instructions. Quantitative real-time PCR was performed by using the SYBR Green real-time PCR Master Mix Reagent Kit (Toyobo, Osaka, Japan). The reactions were performed in a Bio-Rad CFX96 real-time system (Bio-Rad, Hercules, CA, USA) by following the manufacturer's protocol. The amplification reagents contained 10 µL SYBR, 1 µL cDNA, 1.6 µL 10 mM primer pairs, and 7.4 µL RNase-free H2O. All gene-specific primer pairs were designed using online software GenScript, and the optimal primer annealing temperature for each gene is listed in Table S1. The expression of Actin was used as an internal control. The amplification of various genes was normalized by ACTIN-1 expression, and their relative expression levels were calculated by the $2(-\Delta\Delta CT)$ method Wang et al., 2016. The average values and standard errors were calculated from three independent biological replicates

Extraction and Determination of Bioactive Compounds

The determination of carbohydrate was performed by following the method described by Li et al. (2017). The extraction of grain anthocyanin was conducted by using the procedures described previously Yoshimura et al., 2012. The ANS concentration in rice grains was measured spectrophotometrically as previously described by Teng et al. (2005). The ANS amount was calculated as the product of the extraction solution volume and the relative ANS concentration. One ANS unit equals one absorbance unit (A530 − (1/4 × A657)) in 1 mL of extraction solution. Total phenolic content of rice grain was measured according to the method of Dewanto et al., (2002). Total flavonoid content of rice grain was measured according to the method of Zhishen, et al., (1999). The protein content was determined according to Zakari et al., 2020.

Statistical Analysis

All experiments were conducted using a randomized complete block design with three biological replicates per genotype. Data were expressed as means ± standard error (SE).

Agronomic and grain quality traits were compared between the wild-type (9311) and black-kernel mutant (9311 bk) using analysis of variance (ANOVA), followed by least significant difference (LSD) test at $p < 0.05$ for mean separation. For time-course pigment accumulation and gene expression analyses, a two-way ANOVA was performed with genotype and days after anthesis as fixed factors. Where significant interactions were detected, post hoc pairwise comparisons were conducted using Tukey's HSD test. Gene expression was analyzed by Student's *t*-test when comparing two groups or ANOVA when comparing multiple time points. Correlation analyses were performed using Pearson's correlation coefficients (r). Significance levels were tested at $p < 0.05$ and $p < 0.01$. All statistical analyses were conducted using SPSS v25.0 (IBM, Armonk, NY, USA) and graphs were generated with GraphPad Prism v9.0 (GraphPad Software, San Diego, USA).

Results and Discussion

The black-kernel mutant (9311 bk) exhibited notable differences in growth and yield-related traits compared to the wild-type 9311 (Table 1). The mutant plants were significantly taller, with longer panicles and grains, but displayed a reduced seed-setting rate and lower 1000-grain weight. These findings align with earlier reports suggesting that pigment-rich rice genotypes frequently show altered carbon partitioning and reduced assimilate translocation efficiency, leading to yield penalties (Shao et al., 2018; Sun et al., 2019; Zhang et al., 2023). Increased plant height and longer grains may indicate enhanced vegetative vigor or altered hormonal signaling triggered by gamma-ray mutagenesis. Similar trends have been reported in black rice mutants derived from IR64 and Nipponbare backgrounds, where radiation-induced mutations upregulated genes linked to cell elongation but reduced photosynthate allocation to panicle development (Li et al., 2020; Hu et al., 2022). Thus, the present result suggests that anthocyanin pathway activation in 9311 bk may impose a trade-off on reproductive efficiency.

Grain quality assessment revealed that 9311 bk had significantly greater grain length and width but lower brown rice recovery and amylose content compared with the wild type (Table 2). Reduced milling recovery in pigmented rice is consistent with prior observations that thick pericarps and high polyphenolic content compromise endosperm integrity during polishing (Sompong et al., 2011; Goufo and Trindade, 2014). The decrease in amylose content observed here agrees with findings in black and red rice accessions, where enhanced anthocyanin biosynthesis was accompanied by reduced expression of GBSSI (granule-bound starch synthase I), a key enzyme for amylose synthesis (Zhao et al., 2020; Kim et al., 2022). This negative correlation between pigment accumulation and starch biosynthesis may explain the softer texture and higher water absorption typically reported for pigmented rice varieties (Figure 1).

Quantitative biochemical assays revealed that 9311 bk grains accumulated markedly higher total anthocyanidins, flavonoids, and polyphenols than the wild type (Table 3). The upregulation of key anthocyanin biosynthetic genes—PAL, CHS, CHI, F3H, F3′H, DFR, and ANS—was confirmed through qRT-PCR analysis (Figure 2). These genes catalyze sequential steps in the phenylpropanoid pathway, and their co-expression strongly supports enhanced flux toward anthocyanin biosynthesis in the mutant (Tanaka et al., 2010; Qiu et al., 2019; Chen et al., 2020).

Similar transcriptional activation patterns have been documented in black rice cultivars such as Heugjinju and Hom Nin, where increased PAL and DFR expression correlated with higher cyanidin-3-glucoside accumulation (Zhang et al., 2014; Shao et al., 2018). The consistent upregulation of these genes in 9311 bk suggests that gamma-ray

mutagenesis may have induced promoter or regulatory mutations enhancing transcriptional efficiency of the anthocyanin pathway.

Table 1: Variation in important plant phenotypic traits between 9311 bk mutant and its wild type

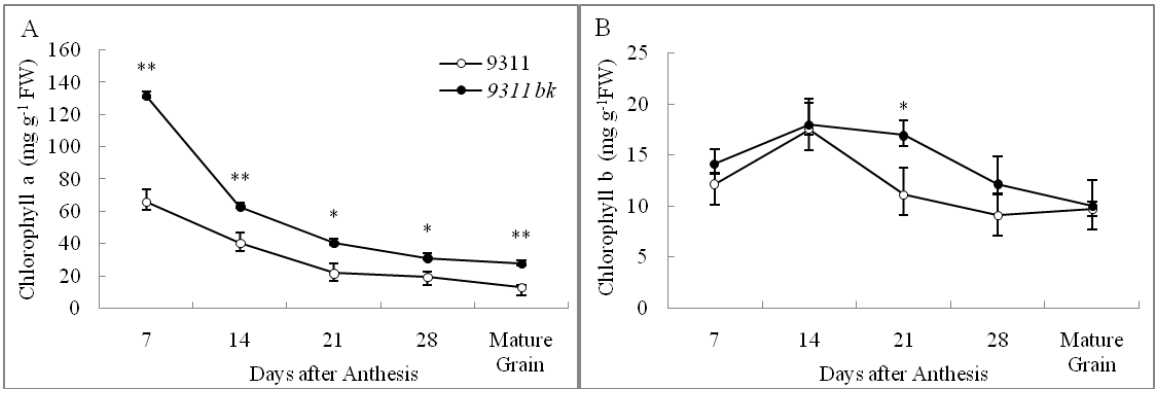
Traits	9311	9311 bk	SD
Plant Height(cm)	118.67 ± 2.2	125.67 ± 1.8	*
Tillers per Plant	7.33 ± 0.8	8.33 ± 0.8	ns
Panicle Length(cm)	24 ± 0.8	24.83 ± 0.5	ns
Grains per Panicle	232 ± 1.4	226 ± 1.9	*
Filled Grains Panicle^{-1}	167.67 ± 1.1	163 ± 0.7	**
Seed Setting Rate	75.23 ± 2.7	67.74 ± 2.0	*
1000-Grain Weight(g)	30.97 ± 0.3	28.5 ± 1.4	**

* & ** Indicates significant difference at 0.05 & 0.01probability level, respectively. ns; not significant

Table 2. Variation in important grain quality traits between 9311 bk mutant and its wild type

Genotype	GL (mm)	GW (mm)	GL / GW	BRR	HMRR	CGR (%)	CD (%)	AC (%)	PC (mg/g)
9311	9.2±0.1	1.96 ±0.0	4.72±0.1	0.77± 0.0	0.53± 0.1	3.82±0.3	41.35± 2.0	27.36± 0.5	2.48 ± 0.1
9311 bk	10± 0.2	2.16 ±0.0	4.65±0.1	0.72± 0.0	0.59± 0.0	2.68±0.9	35.65± 2.8	25.17± 0.8	2.91 ± 0.1
SD	**	**	ns	**	ns	ns	ns	*	*

GL; Grain Length, GW; Grain Weight, BRR; Brown Rice, HMRR; Head Milled Rice Recovery, CD; Chalkiness Degree, AC; Amylose Content, PC; Protein Content



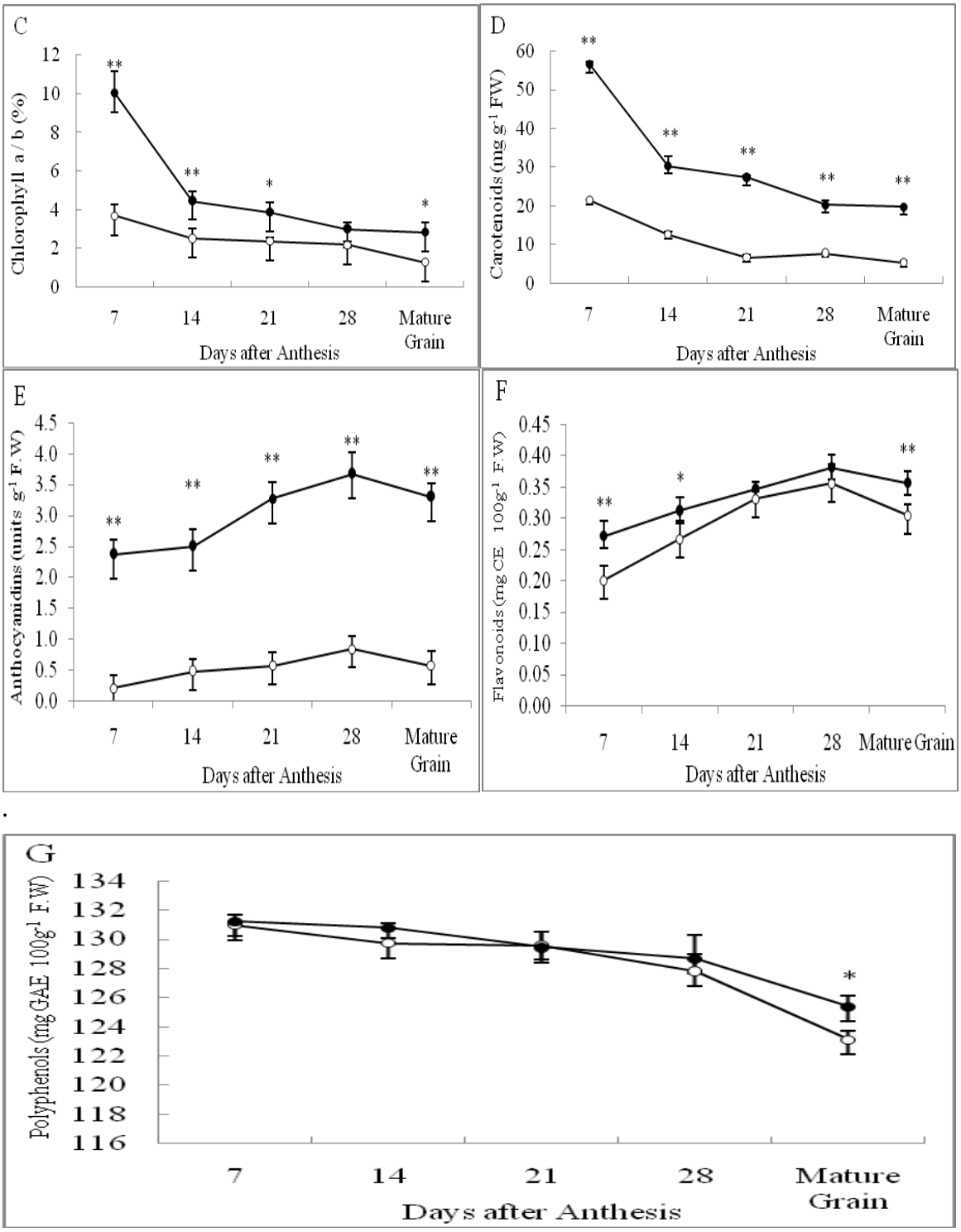


Fig. 1. Temporal patterns of chlorophyll a (A), chlorophyll b (B), chlorophyll a/b (C), carotenoids (D), anthocyanidins (E), flavonoids (F) and polyphenols (G) in grains after anthesis. Vertical bars represent standard errors (n=3). The asterisks represent significant differences

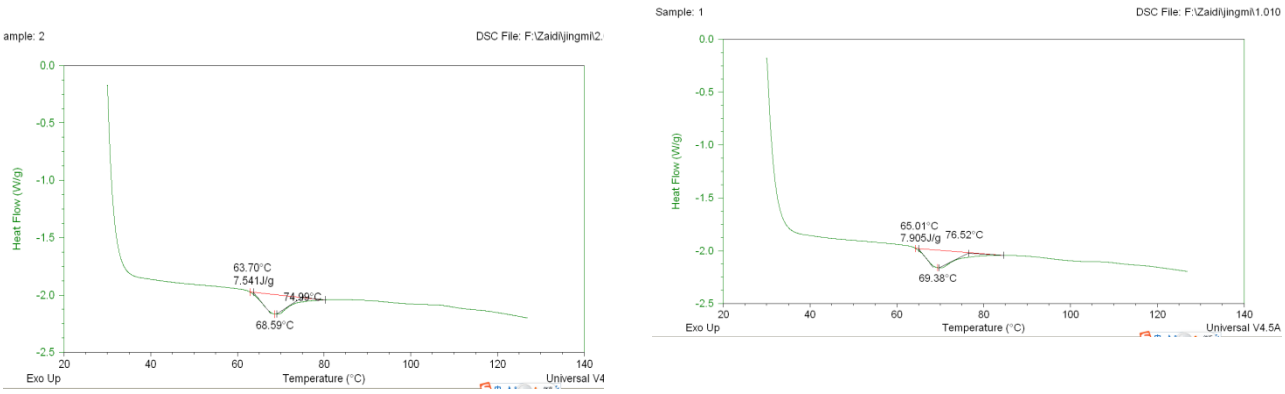


Fig. 2. Heat-flow profiles of mature grains in (A) wild-type 9311 and (B) black-kernel mutant 9311 bk, showing altered starch thermal properties.

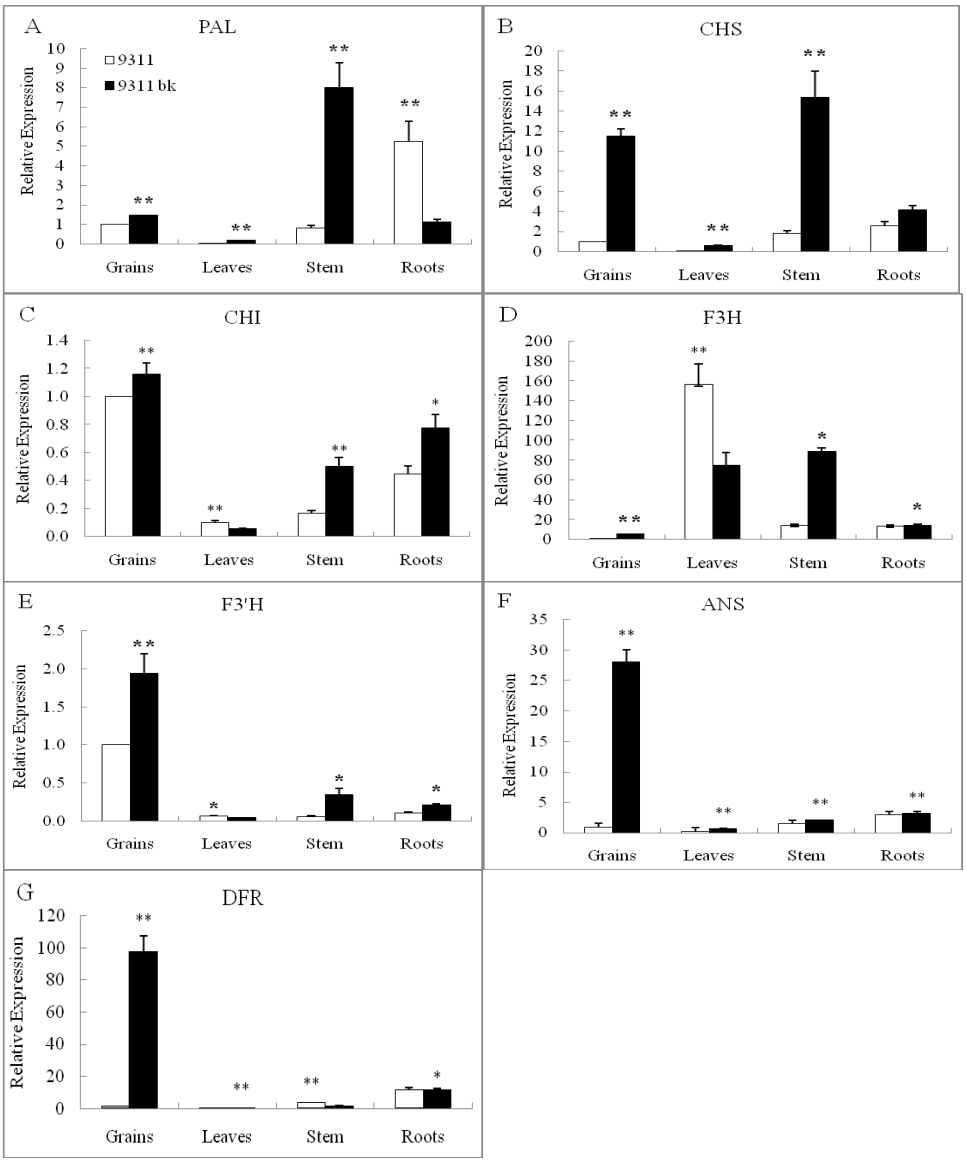


Fig. 2. Transcriptional analysis of seven anthocyanin biosynthesis genes in grain, leaves stem and roots. PAL, phenylalanine ammonia- lyase; CHS, Chalcone synthase; CHI, Chalcone isomerase; F3H, Flavanone 3β-hydroxylase; F3'H, Flavonoid 3'-hydroxylase; ANS, anthocyanidin synthase; DFR, dihydroflavonol 4-reductase. Vertical bars represent standard errors (n=3). The asterisks represent significant differences.

Differential scanning calorimetry (DSC) revealed that the 9311 bk mutant exhibited higher onset (To) and peak (Tp) gelatinization temperatures but lower enthalpy (ΔH) than the wild type (Figure 3). This indicates an altered starch structure, possibly due to reduced amylose-to-amylopectin ratio and differential granule packing, consistent with reports on pigmented rice by Zhang et al. (2021) and Ren et al. (2022). Correlation analyses (Figure 4) showed strong positive associations between anthocyanidin levels and the expression of PAL, CHS, DFR, and ANS, but negative correlations with starch, sucrose, and 1000-grain weight. This suggests that enhanced flux toward flavonoid metabolism diverts photosynthates from carbohydrate biosynthesis—an observation also noted in purple pericarp rice lines (Sun et al., 2019; Hu et al., 2022). The carbohydrate depletion in 9311 bk grains could therefore explain the lower grain weight and amylose content observed.

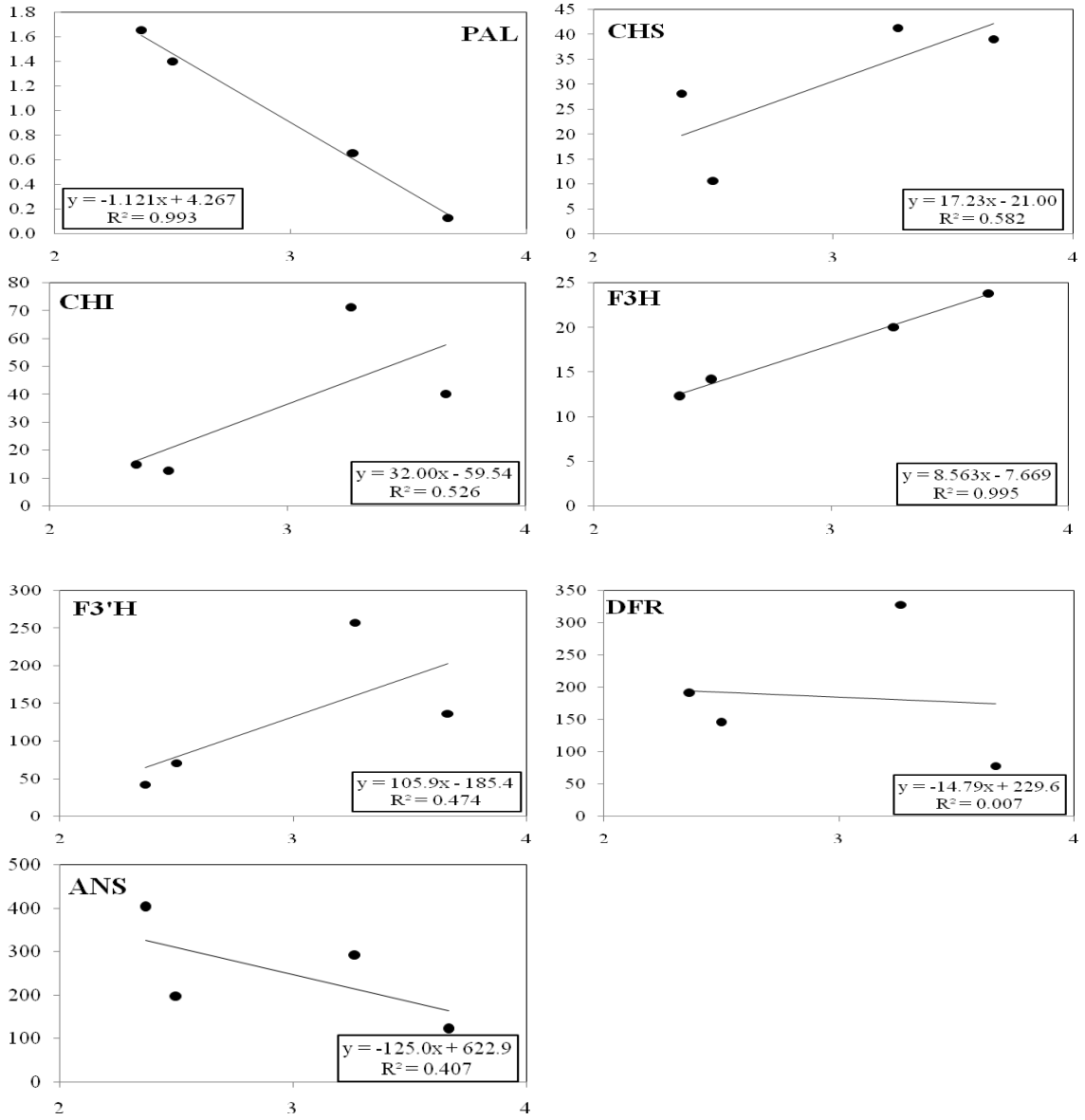


Fig. 3. Correlation between anthocyanidin concentration and expression of anthocyanin biosynthesis genes (*PAL, CHS, CHI, F3H, F3'H, DFR, ANS*) in 9311 bk grains after anthesis.

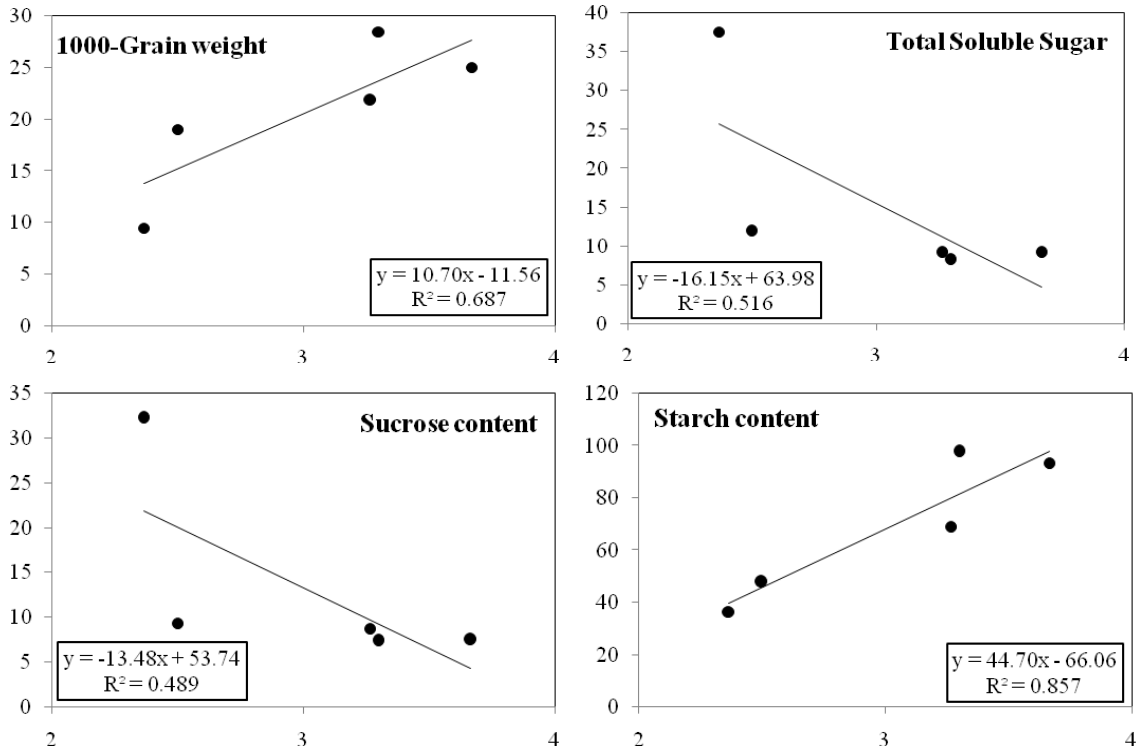


Fig. 4. Correlation between anthocyanidin concentration and 1000-grain weight, soluble sugar, sucrose, and starch content in 9311 bk after anthesis.

The concurrent enhancement of anthocyanin content and decline in yield parameters underscores a critical metabolic trade-off. As flavonoid biosynthesis competes with starch biosynthesis for common precursors (e.g., glucose-6-phosphate and phosphoenolpyruvate), higher pigment accumulation inevitably constrains carbohydrate deposition during grain filling (Shao et al., 2018; Kim et al., 2022). Nevertheless, from a functional food perspective, the elevated anthocyanin and polyphenol levels in 9311 bk present valuable nutritional and health-promoting traits, offering antioxidant and anti-inflammatory benefits (Ling et al., 2021; Zhang et al., 2023). Thus, future breeding efforts should focus on balancing anthocyanin accumulation with improved sink strength and starch metabolism to mitigate yield penalties.

Conclusion

This study offers integrated physiological, biochemical, and molecular evidence that anthocyanin buildup in pigmented rice is closely linked to the activation of the flavonoid biosynthetic pathway, while also showing measurable trade-offs in yield and grain quality. The gamma-ray-induced black-kernel mutant (9311 bk) exhibited strong upregulation of PAL, CHS, CHI, F3H, F3'H, DFR, and ANS genes, leading to significantly higher levels of anthocyanidin, flavonoid, and polyphenol contents. However, this boost in secondary metabolism was accompanied by reductions in seed-setting rate, grain weight, and amylose content, along with changes in starch thermal properties, indicating a metabolic competition between carbon flows toward pigment biosynthesis and starch accumulation during grain filling. Therefore, our results not only confirm the molecular connection between anthocyanin synthesis and carbohydrate metabolism but also highlight the complexity of balancing nutritional quality with agronomic performance in rice. These insights can guide molecular breeding and CRISPR-based strategies aimed at fine-tuning transcriptional regulators (e.g., OsC1, Rc, bHLH1) to develop pigmented rice cultivars combining high

nutritional value with agronomic stability. Such balanced varieties could support the growing demand for functional foods and sustainable rice production systems.

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