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Reinterpretation of anthocyanins biosynthesis in developing black rice seeds through gene expression analysis

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Abstract

The biosynthesis of anthocyanins is still questionable in regulating the quantities of anthocyanins biosynthesized in rice seeds and the expression levels of transcription factors and the structural genes involved in the biosynthetic pathway of anthocyanins. We herein investigated the relationship between the accumulated anthocyanin contents and the expression levels of genes related to the biosynthesis of anthocyanins in rice seeds. Liquid chromatography/mass spectrometry-mass spectrometry analysis of cyanidin 3-glucoside (C3G) in rice seeds showed no accumulation of C3G in white and red rice cultivars, and the differential accumulation of C3G among black rice cultivars. RNA-seq analysis in rice seeds, including white, red, and black rice cultivars, at twenty days after heading (DAH) further exhibited that the genes involved in the biosynthesis of anthocyanins were differentially upregulated in developing seeds of black rice. We further verified these RNA-seq results through gene expression analysis by a quantitative real-time polymerase chain reaction in developing seeds of white, red, and black rice cultivars at 20 DAH. Of these genes related to the biosynthesis of anthocyanins, *bHLH*s, *MYB*s, and *WD40*, which are regulators, and the structural genes, including *chalcone synthase* (*CHS*), *flavanone 3-hydroxylase* (*F3H*), *flavonoid 3´-hydroxylase* (*F3´H*), *dihydroflavonol 4-reductase* (*DFR*), and *anthocyanidin synthase* (*ANS*), were differentially upregulated in black rice seeds. The correlation analysis revealed that the quantities of C3G biosynthesized in black rice seeds were positively correlated to the expression levels of *bHLH*s, *MYB*s and *WD40*, *CHS*, *F3H*, *F3´H*, *DFR*, and *ANS*. In addition, we present *bHLH2* (*LOC_Os04g47040*) and *MYB*s (*LOC_Os01g49160*, *LOC_Os01g74410*, and *LOC_Os03g29614*) as new putative transcription factor genes for the biosynthesis of anthocyanins in black rice seeds. It is expected that this study will help to improve the understanding of the molecular levels involved in the biosynthesis of anthocyanins in black rice seeds.

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Introduction

Anthocyanins, a class of flavonoids [1], have been identified in various plant species with their specific anthocyanin(s), i.e., malvidin 3-galactoside in *Primula polyanthus* [2], peonidin 3-glucoside in *Oxycoccus macrocarpus* [3], pelargonidin 3-glucoside in *Fragaria chiloensis* [4], cyanidin 3-glucoside in *Rumex crispus* [5] and *Spirodela intermedia* [6], glucosides of cyanidin, delphinidin, malvidin and petunidin in *Medicago sativa* [7], cyanidin 3-glucoside, peonidin 3-glucoside, cyanidin 3-rutinoside and cyanidin 3-galactoside in *Oryza sativa* [8–10]. It has been known that anthocyanins are induced in plants by biotic [11] or abiotic [5, 12, 13] stress and have an antioxidative activity [14, 15].

In the analysis of anthocyanins in various organ/tissue samples, including leaf blade, leaf sheath, collar, internode, auricle, ligule, hull, apiculus, and pericarp, of Purpleputtu, a black rice cultivar, Reddy et al. reported cyanidin as a major anthocyanidin and peonidin, a 3′-methoxy cyanidin derivative as a minor anthocyanidin [8]. Yoshimura et al. exhibited that black rice seeds contain cyanidin 3-glucoside (C3G), a major anthocyanin, and peonidin 3-glucoside (P3G), a minor anthocyanin. They accumulate in the outer pericarp and seed coat layer of black rice seeds. They further reported various glucosides of cyanidin, peonidin (3′-methoxy cyanidin), petunidin (3′-hydroxy-5′-methoxy cyanidin), and malvidin (3′,5′-dimethoxy cyanidin) [10].

It was first known in *Zea mays* that the biosynthesis of anthocyanins is regulated by two transcription factors, including *C1* [16, 17], a myb gene, and *R* [18], a basic helix-loop-helix (bHLH) gene [19]. Orthologous genes to these two genes were also reported in rice [20, 21]. The biosynthetic pathway of anthocyanins has been well elucidated in plants, as presented in Fig 1. The first step of the biosynthesis of anthocyanins is the conversion of *p*-coumaroyl CoA, formed from phenylalanine via stepwise reactions by phenylalanine ammonia-lyase (PAL) [22], cinnamate 4-hydroxylase (C4H) [23, 24], and 4-coumarate: CoA ligase (4CL) [25], respectively, and malonyl CoA into naringenin chalcone by chalcone synthase (CHS) [26, 27]. Naringenin chalcone is finally converted into several kinds of anthocyanins via several reactions by chalcone isomerase (CHI) [28–30], flavanone 3-hydroxylase (F3H) [31, 32], dihydroflavonol 4-reductase (DFR) [33–35], flavonoid 3′-hydroxylase (F3′H) [36, 37], flavonoid 3′,5′-hydroxylase (F3′5′H) [38], anthocyanidin synthase (ANS) [39–41], and UDP-glucose: anthocyanidin 3-*O*-glucosyltransferase (A3GT) [40, 42, 43], respectively (Fig 1).

Compared to developing seeds of white rice with no anthocyanin accumulation, *CHS*, *F3H*, *DFR*, and *ANS* were upregulated in developing black rice seeds [44] and *Kala4* (*LOC_Os04g47059*), an orthologous *bHLH* gene of maize *R* gene, was also upregulated in black rice seeds [45]. Among black rice cultivars, there were significant differences in the quantities of anthocyanins, C3G and P3G, in mature rice seeds [46]. However, in developing seeds of black rice, there needs to be more information on the relationship between the quantities of anthocyanins biosynthesized and the expression level of genes related to the biosynthetic pathway of anthocyanins. In this study, we investigated how the biosynthesis of anthocyanins is related to the expression of genes involved in the biosynthetic pathway of anthocyanins in developing seeds of black rice.

Materials and methods

Growth of rice cultivars used in this study

We transplanted and cultivated three replicates of seedlings of Dongjin (white rice), Geonganghongmi (red rice), Jeokjinju (red rice), Boseokheukchal (black rice), Heukjinju (black rice), Heukjinmi (black rice), and Heukseol (black rice) in the experimental paddy field of the

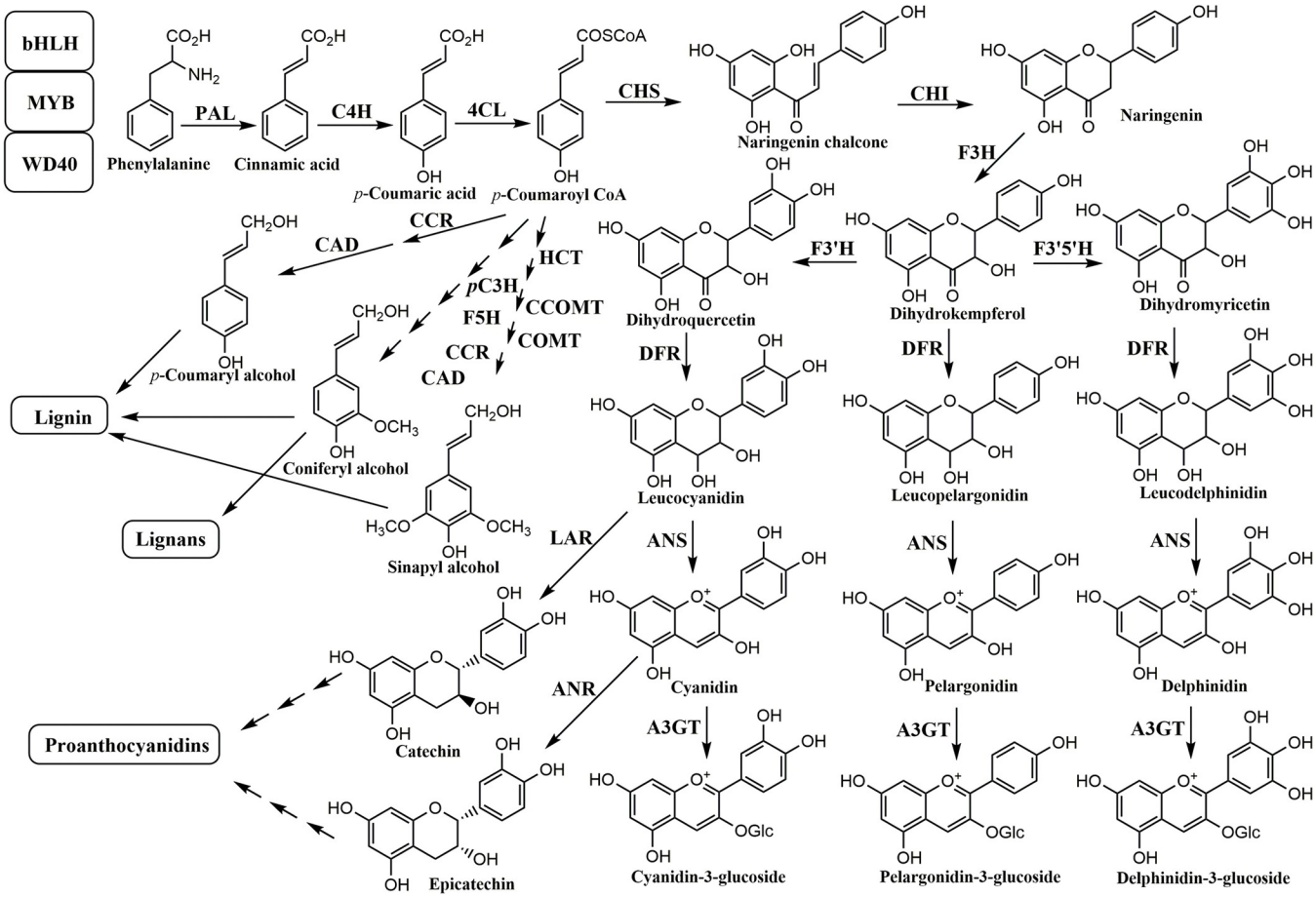

Fig 1. Schematic representation of the biosynthetic pathway of anthocyanins in plants. bHLH: basic helix-loop-helix protein; MYB: myb protein; WD40: tryptophan-aspartic acid repeat protein; PAL: phenylalanine ammonia-lyase; C4H: cinnamate 4-hydroxylase; 4CL: 4-coumarate: CoA ligase; CCR: cinnamoyl-CoA reductase; CAD: cinnamyl alcohol dehydrogenase; HCT: hydroxycinnamoyl-CoA shikimate/quinate hydroxycinnamoyltransferase; *p*C3H: *p*-coumarate 3-hydroxylase; CCOMT: caffeoyl-CoA *O*-methyltransferase; COMT: caffeate *O*-methyltransferase; F5H: ferulate 5-hydroxylase; CHS: chalcone synthase; CHI: chalcone isomerase; F3H: flavanone 3-hydroxylase; DFR: dihydroflavonol 4-reductase; F3′H: flavonoid 3′-hydroxylase; F3′5′H: flavonoid 3′,5′-hydroxylase; ANS: anthocyanidin synthase; LAR: leucoanthocyanidin reductase; ANR: anthocyanidin reductase; A3GT: UDP-glucose: anthocyanidin 3-*O*-glucosyltransferase.

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National Institute of Crop Science (NICS), Republic of Korea, by a completely randomized design using the standard rice cultivation method of the NICS. We sampled developing seeds from one panicle per plant and four plants per replicate at twenty days after heading (DAH), and harvested mature seeds at around sixty DAH.

Quantification of cyanidin 3-glucoside by liquid chromatography/ mass spectrometry-mass spectrometry

We quantified cyanidin 3-glucoside (C3G) content in the flour of hulled rice with three replicates of Dongjin, Geonganghongmi, Jeokjinju, Boseokheukchal, Heukjinju, Heukjinmi, and Heukseol using the methods developed in the Center for University-Wide Research Facilities at the Jeonbuk National University with liquid chromatography/mass spectrometry-mass spectrometry [LC/MS-MS, Xevo TQ-S triple quadrupole mass spectrometer (Waters Corporation, Milford, USA) coupled with ACQUITY Ulta-Performance Liquid chromatography system (Waters Corporation, Milford, MA, USA)] [47].

We purchased the standard material, cyanidin 3-glucoside, from Sigma-Aldrich (Saint Louis, MO, USA).

Total RNA extraction and RNA-seq

We extracted total RNA from the frozen and milled samples with three replicates of developing seeds of Dongjin, Geonganghongmi, Jeokjinju, Boseokheukchal, Heukjinju, Heukjinmi, and Heukseol at 20 DAH using the RNeasy Plant Mini Kit (QIAGEN, Hilden, Germany) with the manufacturer's instructions. Of these total RNA samples, we sent each one replicate of the total RNA samples of Dongjin (white rice), Jeokjinju (red rice), and Heukseol (black rice) to Macrogen, Inc (Seoul, Republic of Korea) for RNA-seq using the Illumina technology by paired-end type sequencing with 101-bp read length.

We processed the raw data of RNA-seq by the methods described by Lee et al. [48]. Briefly, the raw data were quality trimmed using the Cutadapt software with parameters: `-a AGATCGGAAGAGC-A AGATCGGAAGAGC`–q 30 –m 20 [49], and the trimmed data were mapped to a reference rice genome, MSU7 (http://rice.uga.edu/) using the HISAT2 software [50] with default parameter (S1 Table). Read counts data were calculated with the featureCounts software [51] (S2 Table). We finally obtained normalized read counts data from the processed raw data of RNA-seq by division of the read counts of all genes with those of the *OsUBI1* gene (*LOC_Os03g13170*) (S2 Table). The raw data of RNA-seq are available at https://www.ebi.ac.uk/arrayexpress/experiments/E-MTAB-9993.

Gene expression analysis by quantitative real time-polymerase chain reaction

As described by Lee et al. [48], we synthesized cDNA using the iScript^{TM} cDNA Synthesis Kit (Bio-Rad, Hercules, CA, USA) from 1 μg of each total RNA taken in total RNA samples with three replicates of Dongjin, Geonganghongmi, Jeokjinju, Boseokheukchal, Heukjinju, Heukjinmi, and Heukseol. We carried out quantitative real time-polymerase chain reaction (qRT-PCR) with cDNA and the primer sets listed in the S3 Table in the CFX96^{TM} Real-Time Detection System (Bio-Rad, Hercules, CA, USA) using iQ SYBR Green Supermix (Bio-Rad, Hercules, CA, USA). We used the *OsUBI1* gene, *LOC_Os03g13170*, as a reference gene (S3 Table) [52–54]. We used the Pfaffl [55] method to determine the relative expression of genes described in the S3 Table.

Generation of heatmap

We generated all heatmaps with $\log_2$(FPKM+1) values using the pheatmp package in R version 1.2.5033 (Kolde, 2018; RRID:SCR_016418). We obtained FPKM values for various rice organs of Nipponbare (white rice) from the Rice Genome Annotation Project (http://rice.uga.edu/).

Statistical analysis

We performed an analysis of variance (ANOVA) and Duncan's Multiple Range Test (DMRT) using SAS9.4 TS Level 1 M5 (Ver.1.0.19041; SAS Institute Inc., Cary, NC, United States). We used the package corrplot in R version 1.2.5033 to conduct correlation analysis [56].

Results

Quantification of cyanidin 3-glucoside in seeds of black rice cultivars through LC/MS-MS

The National Institute of Crop Science (NICS) has developed and released fourteen black rice cultivars (S3 Table). Of these fourteen black rice cultivars, we selected four black rice cultivars, including Boseokheukchal, Heukjinju, Heukjinmi, and Heukseol, with consideration of quantities of C3G in hulled seeds and original parent(s) used in the breeding program for improvement of black rice traits. Interestingly, the genome of Heukjinmi partially retains the genomic content of Hongjinju, a red rice cultivar, as one of the parents in its breeding pedigree (S4 Table, http://www.nics.go.kr/api/breed.do?m=100000128&homepageSeCode=nics). We also selected one white rice cultivar, Dongjin, and two red rice cultivars, Geonganghongmi and Jeokjinju, as a control to compare the metabolic differences with black rice cultivars from the database for cultivars developed by the NICS (http://www.nics.go.kr/api/breed.do?m=100000128&homepageSeCode=nics).

We quantified C3G from the hulled seeds of seven rice cultivars, including Dongjin, Geonganghongmi, Jeokjinju, Boseokheukchal, Heukjinju, Heukjinmi, and Heukseol, through liquid chromatography/mass spectrometry-mass spectrometry (LC/MS-MS) (Fig 2). Of seven rice cultivars used in this study, C3G was detected only in the hulled seeds of black rice cultivars. Based on the quantities of C3G, four black rice cultivars are statistically classified into two groups; Boseokheukchal and Heukjinmi; Heukjinju and Heukseol. The quantities of C3G in the hulled seeds of Heukjinju and Heukseol were significantly higher than those in the hulled seeds of Boseokheukchal and Heukjinmi (Fig 2).

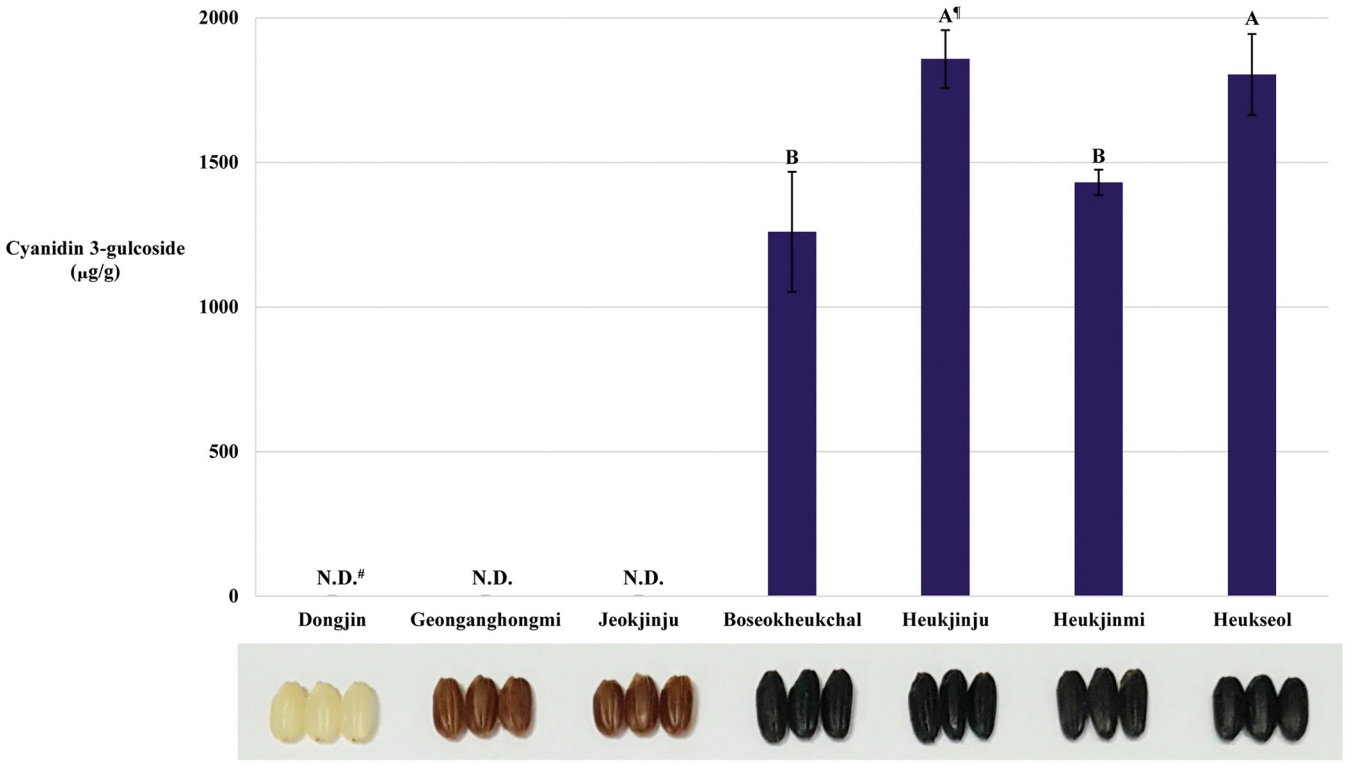

Fig 2. Liquid chromatography-mass spectrometry/mass spectrometry analysis of cyanidin 3-glucoside in hulled seeds of pigmented rice cultivars. Dongjin, Geonganghongmi, Jeokjinju, Boseokheukchal, Heukjinju, Heukjinmi, and Heukseol. ¶: Duncan's Multiple Range Test (DMRT, $\alpha = 0.05$) was performed after the confirmation of statistical significance for these data through analysis of variance (ANOVA). #N.D.: not detected. The data represents mean ± standard deviation (SD).

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Genes, involved in the biosynthetic pathway of anthocyanins, detected in developing black rice seeds through RNA-seq

We confirmed that the quantity of C3G and P3G maximally accumulated in the seeds of Heuknam and Heukseol, black rice cultivars, at around 20 DAH (Lee *et al*., unpublished), which corresponds to a previous report [57], and collected developing seeds of Dongjin, Geonganghongmi, Jeokjinju, Boseokheukchal, Heukjinju, Heukjinmi, and Heukseol at 20 DAH for gene expression analysis. To check the overall expression patterns of genes putatively involved in the biosynthetic pathway of anthocyanins, including *bHLH* [21, 45], *MYB* [20, 58], *WD40* [59], *PAL* [22], *C4H* [23, 24], *4CL* [25], *CHS* [26, 27], *CHI* [28–30], *F3H* [31, 32], *F3´H* [36, 37], *DFR* [33–35], and *ANS* [39–41], and other genes indirectly related to the biosynthetic pathway of anthocyanins, including *HCT* [60], *CCR* [61, 62], *CAD* [63], and *LAR* [64], in developing seeds of white, red, and/or black rice at 20 DAH, only one replicate of total RNA samples of Dongjin, Jeokjinju, or Heukseol was chosen for RNA-seq through Illumina sequencing. These RNA-seq data were used to identify potential candidate genes involved in the biosynthetic pathway of anthocyanins.

We detected a total 34,290 genes in developing rice seeds of Dongjin, Jeokjinju and/or Heukseol at 20 DAH after analysis of raw data of RNA-seq (S2 Table) and, based on amino acid sequence homology with each reference gene, i.e. *bHLH* [21, 45], *MYB* [20, 58], *WD40* [59], *PAL* [22], *C4H* [23, 24], *4CL* [25], *CHS* [26, 27], *CHI* [28–30], *F3H* [31, 32], *F3´H* [36, 37], *DFR* [33–35], *ANS* [39–41], *HCT* [60], *CCR* [61, 62], *CAD* [63], and *LAR* [64], searched and selected orthologous gene(s) for each reference gene (S2 and S5 Tables). For these candidate genes, the verification was intensively performed through qRT-PCR with more than three biological replicates.

Fifty-four bHLH genes and seventy-five MYB genes were detected in developing seeds of Dongjin, Jeokjinju and/or Heukseol at 20 DAH (S5 Table). Among these genes, three *bHLH*s—*LOC_Os01g09990*, *LOC_Os04g47040*, and *LOC_Os04g47059* (known as *OSB2* or *Kala4*)—and three *MYB*s—*LOC_Os01g49160*, *LOC_Os01g74410*, and *LOC_Os03g29614*—only showed preferential upregulation in developing seeds of Heukseol, compared to those of Dongjin and Jeokjinju. Moreover, of homologs for each gene, we could confirm that *WD40* (*LOC_Os02g45810*), *CHS* (*LOC_Os11g32650*), *CHI* (*LOC_Os03g60509*), *F3H* (*LOC_Os04g56700*), *F3´H* (*LOC_Os10g17260*), *DFR* (*LOC_Os01g44260*), and *ANS* (*LOC_Os01g27490*) were also preferentially upregulated in developing seeds of Heukseol. Predominantly, *ANS* (*LOC_Os01g27490*) was only expressed in developing seeds of Heukseol, but not in those of Dongjin and Jeokjinju (S5 Table).

Of these genes in S5 Table, based on their preferential expression patterns in the developing seeds of Heukseol, a black rice cultivar, we selected genes putatively involved in the biosynthetic pathway of anthocyanins, i.e., *bHLH1*, *LOC_Os04g47059*; *bHLH2*, *LOC_Os04g47040*; *MYB*, *LOC_Os01g49160*; *WD40*, *LOC_Os02g45810*; *PAL*, *LOC_Os02g41630*; *C4H*, *LOC_Os05g25640*; *4CL*, *LOC_Os02g08100*; *HCT*, *LOC_Os04g42250*; *CCR*, *LOC_Os09g25150*; *CAD*, *LOC_Os02g09490*; *CHS*, *LOC_Os11g32650*; *CHI*, *LOC_Os03g60509*; *F3H*, *LOC_Os04g56700*; *F3´H*, *LOC_Os10g17260*; *DFR*, *LOC_Os01g44260*; *ANS*, *LOC_Os01g27490*; and *LAR*, *LOC_Os03g15360*, to verify their expression in developing seeds of Dongjin, Geonganghongmi, Jeokjinju, Boseokheukchal, Heukjinju, Heukjinmi, and Heukseol at 20 DAH by qRT-PCR (S1 Fig and S5 Table). As mentioned above, these genes, except for *4CL* (*LOC_Os02g08100*), *HCT* (*LOC_Os04g42250*), *CCR* (*LOC_Os09g25150*), *CAD* (*LOC_Os02g09490*), and *LAR* (*LOC_Os03g15360*), showed preferential upregulation in developing seeds of Heukseol, compared to those of Dongjin and Jeokjinju (S1 Fig and S5 Table). We further investigated the expression levels of genes related to the biosynthesis of anthocyanins from RNA-seq data of Nipponbare

(white rice) in the Rice Genome Annotation Project (http://rice.uga.edu/) to support our RNA-seq data (S2 Fig). As shown in developing seeds of Dongjin (S1 Fig and S5 Table), several genes, including *bHLH*s (*LOC_Os04g47040* and *LOC_Os04g47059*), *MYB* (*LOC_Os01g49160*), *WD40* (*LOC_Os02g45810*), *CHS* (*LOC_Os11g32650*), *CHI* (*LOC_Os03g60509*), *F3H* (*LOC_Os04g56700*), *F3´H* (*LOC_Os10g17260*), *DFR* (*LOC_Os01g44260*), and *ANS* (*LOC_Os01g27490*), upregulated in developing seeds of Heukseol, a black rice cultivar, were not upregulated in Nipponbare seed samples, including seeds at ten days after pollination (DAP), the embryo at 25 DAP and endosperm at 25 DAP, but, in Nipponbare 5 DAP seeds, *MYB* (*LOC_Os01g49160*), *WD40* (*LOC_Os02g45810*), *F3H* (*LOC_Os04g56700*), and *DFR* (*LOC_Os01g44260*) were up-regulated, compared to other genes (S2 Fig). *bHLH1* (*LOC_Os04g47059*) was upregulated only in seedling_leaf and anther of Nipponbare, and *ANS* (*LOC_Os01g27490*) was upregulated in anther (S2 Fig).

Verification of the expression of genes involved in the biosynthetic pathway of anthocyanins in RNA-seq data of developing black rice seeds

In developing seeds of Dongjin, Geonganghongmi, Jeokjinju, Boseokheukchal, Heukjinju, Heukjinmi, and Heukseol at 20 DAH, we verified the expression of genes in the phenylpropanoid pathway, i.e., *PAL* (*LOC_Os02g41630*) [22], *C4H* (*LOC_Os05g25640*) [23, 24], *4CL* (*LOC_Os02g08100*) [25], *HCT* (*LOC_Os04g42250*) [60], *CCR* (*LOC_Os09g25150*) [61, 62], and *CAD* (*LOC_Os02g09490*) [63] through qRT-PCR (Fig 1, S3 Fig and S4 Table). These genes were classified into two groups: *PAL*, *C4H*, and *4CL*, which biosynthesize *p*-coumaroyl CoA, an intermediate in phenylpropanoid pathway [65] and a precursor in the biosynthetic pathway of flavonoids [26, 27], including anthocyanins; *HCT*, *CCR*, and *CAD*, genes in a branching point from *p*-coumaroyl CoA toward the biosynthetic pathway of monolignols [60, 62, 63]. As a result of the expression analysis for these genes in the phenylpropanoid pathway, we did not identify any apparent black rice-specific expression patterns in developing seeds at 20 DAH (S3 Fig).

Putative regulator genes, including *bHLH1* (*LOC_Os04g47059*), *bHLH2* (*LOC_Os04g47040*), *MYB* (*LOC_Os01g49160*), and *WD40* (*LOC_Os02g45810*), and structural genes, including *CHS* (*LOC_Os11g32650*), *F3H* (*LOC_Os04g56700*), *F3´H* (*LOC_Os10g17260*), and *DFR* (*LOC_Os01g44260*), in the biosynthetic pathway of anthocyanins were explicitly upregulated in black rice seeds at 20 DAH (Fig 3). Especially, of two *bHLH* genes, *bHLH1* (*LOC_Os04g47059*) showed much higher relative expression in black rice seeds than *bHLH2* (*LOC_Os04g47040*). Although for these genes, Geonganghongmi and Jeokjinju, red rice cultivars, exhibited significantly lower expression levels in seeds at 20 DAH than those of black rice cultivars, these red rice cultivars had much higher expression levels in their seeds, compared to Dongjin, a white rice cultivar. Moreover, four genes, including *bHLH2* (*LOC_Os04g47040*), *WD40* (*LOC_Os02g45810*), *F3H* (*LOC_Os04g56700*) and *DFR* (*LOC_Os01g44260*), showed statistically significant upregulation in the seeds of red rice than those of white rice (Fig 3). Interestingly, *CHI* (*LOC_Os03g60509*), located between *CHS* (*LOC_Os11g32650*) and *F3H* (*LOC_Os04g56700*) in the biosynthetic pathway of anthocyanins [26–32], did not exhibit black rice-specific upregulation in its expression but seemed to have significantly higher expression in the seeds of red and black rice cultivars than those of white rice (S4 Fig).

We verified that *ANS* (*LOC_Os01g27490*) showed black rice-specific expression patterns in seeds at 20 DAH, but it showed no expression in white rice (Dongjin) and red rice (Geonganghongmi and Jeokjinju) seeds (Fig 4), as shown in S5 Table. Among black rice cultivars, there were statistical differences in the expression of *ANS* (*LOC_Os01g27490*) (Fig 4). Moreover, we investigated the expression levels of *LAR* (*LOC_Os03g15360*), which converts leucocyanidin into catechin, one of the red rice-specific compounds [64, 66], in developing seeds of Dongjin, Geonganghongmi, Jeokjinju, Boseokheukchal, Heukjinju, Heukjinmi, and Heukseol at 20

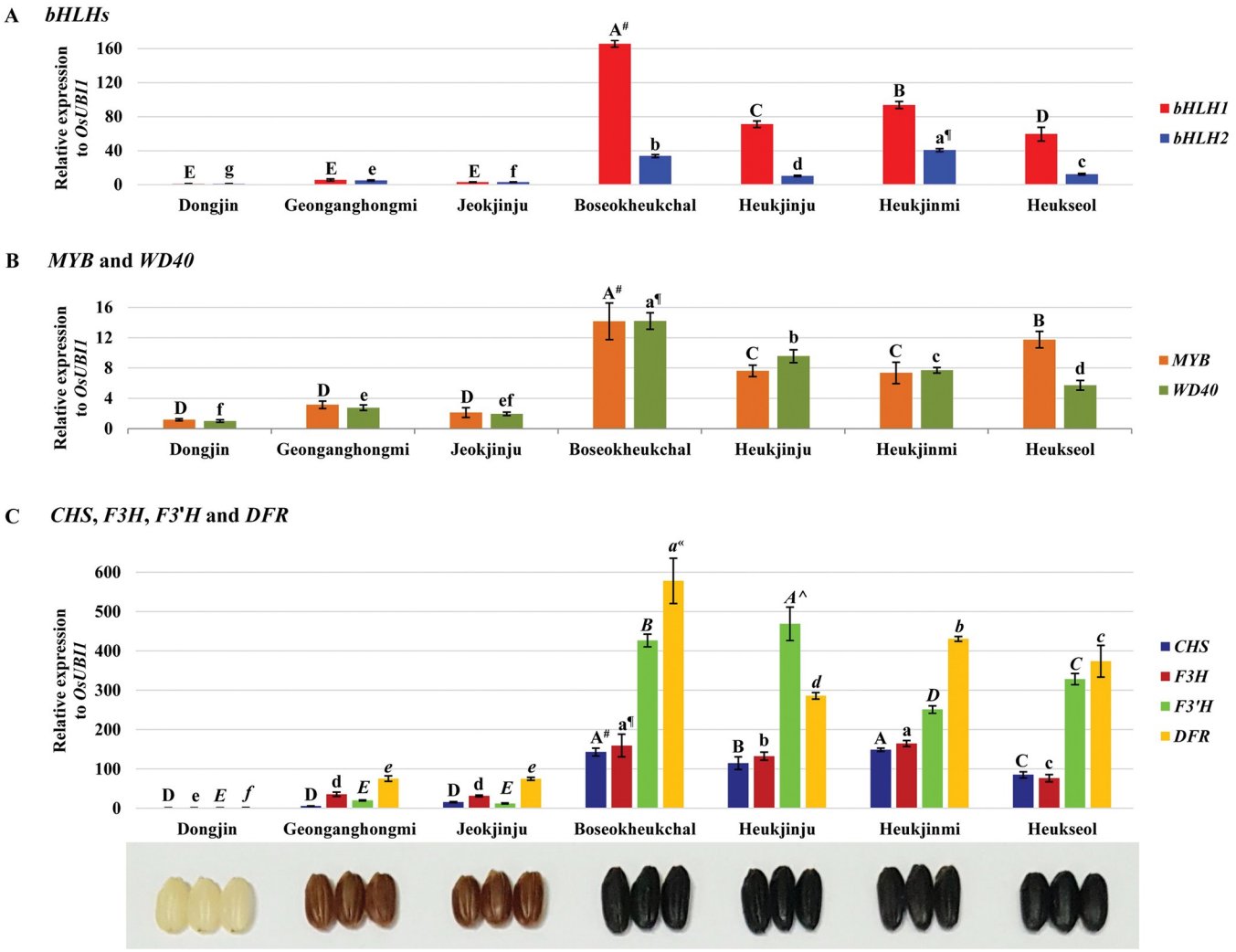

Fig 3. The relative expression of differentially upregulated genes in the biosynthetic pathway of anthocyanins in developing seeds of black rice cultivars, Boseokheukchal, Heukjinju, Heukjinmi, and Heukseol at twenty days after heading through q quantitative real-time polymerase chain reaction. A. *bHLH1*: *LOC_Os04g47059*, *bHLH2*: *LOC_Os04g47040*; **B.** *MYB*: *LOC_Os01g49160*, *WD40*: *LOC_Os02g45810*; **C.** *CHS*: *LOC_Os11g32650*, *F3H*: *LOC_Os04g56700*, *F3'H*: *LOC_Os10g17260*, *DFR*: *LOC_Os01g44260*. The data represents mean ± standard deviation (SD). #, ¶, ^, “: Duncan’s Multiple Range Test (DMRT, α = 0.05) was performed after the confirmation of statistical significance for each dataset through analysis of variance (ANOVA).

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DAH, because Heukjinmi (black rice) has red rice as one of the parents as mentioned above. Of these rice cultivars, Geonganghongmi (red rice) has the maximum expression of *LAR* (*LOC_Os03g15360*) in its seeds, and Jeokjinju (red rice), Boseokheukchal (black rice) and Heukjinmi (black rice) also showed statistically significant upregulation of *LAR* (*LOC_Os03g15360*) in their seeds, compared to Dongjin (white rice), Heukjinju (black rice), and Heukseol (black rice) (Fig 4).

Correlation analysis between the quantity of cyanidin 3-glucoside and the expression level of each gene involved in the biosynthetic pathway of anthocyanins in developing rice seeds

We performed a correlation analysis in Dongjin, Geonganghongmi, Jeokjinju, Boseokheukchal, Heukjinju, Heukjinmi, and Heukseol, to investigate the statistical relationship in the

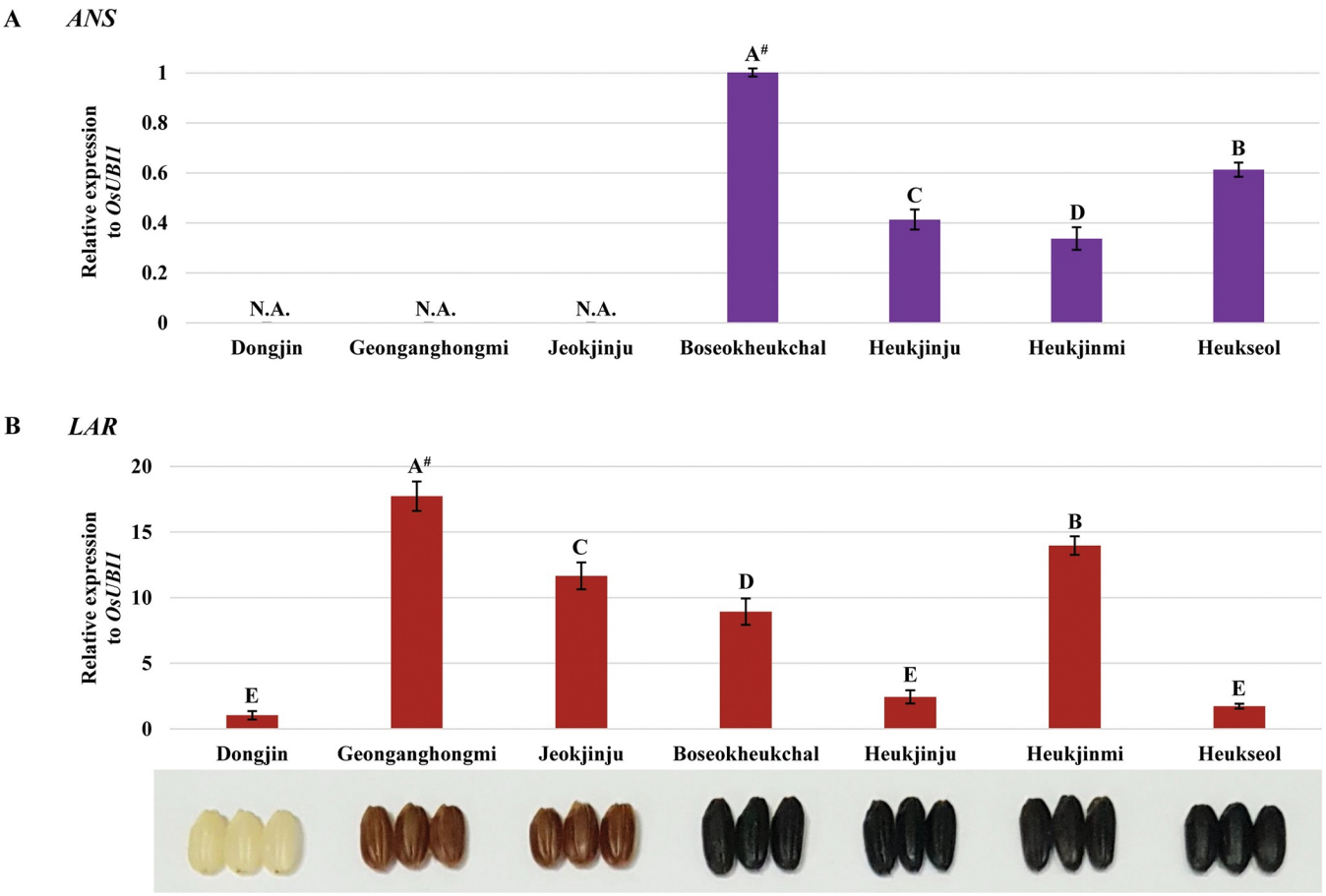

Fig 4. Relative expression of *ANS* (A, *LOC_Os01g27490*) and *LAR* (B, *LOC_Os03g15360*), respectively, in developing seeds of Dongjin, Geonganghongmi, Jeokjinju, Boseokheukchal, Heukjinju, Heukjinmi and Heukseol at twenty days after heading through a quantitative real-time polymerase chain reaction. The data represents mean ± standard deviation (SD). #: Duncan's Multiple Range Test (DMRT, $\alpha = 0.05$) was performed after the confirmation of statistical significance for each dataset through analysis of variance (ANOVA). N.A.: not applicable.

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quantity of C3G in hulled rice seeds and the expression levels of each gene putatively involved in the biosynthetic pathway of anthocyanins in rice seeds at 20 DAH (Fig 5 and S6 Table). The quantity of C3G in hulled rice seeds is positively correlated to the expression of these genes, including *bHLH1* (*LOC_Os04g47059*), *bHLH2* (*LOC_Os04g47040*), *MYB* (*LOC_Os01g49160*), *WD40* (*LOC_Os02g45810*), *CHS* (*LOC_Os11g32650*), *F3H* (*LOC_Os04g56700*), *F3′H* (*LOC_Os10g17260*), *DFR* (*LOC_Os01g44260*), and *ANS* (*LOC_Os01g27490*), preferentially upregulated in black rice seeds as described in Figs 3 and 4. These genes also had a positive correlation between their expression values. Interestingly, the expression of *CHI* (*LOC_Os03g60509*) did not correlate with the quantity of C3G. However, it positively correlated with the expression of genes preferentially upregulated in black rice seeds mentioned above. In addition, the expression of *CCR* (*LOC_Os09g25150*), which shares *p*-coumaroyl CoA as a precursor with *CHS* (*LOC_Os11g32650*) [26, 27, 61, 62], was negatively correlated with the quantity of C3G (Fig 5 and S6 Table).

Discussion

In rice, C3G and P3G were reported as major and minor anthocyanins, respectively [8, 10, 46], and anthocyanins were differentially biosynthesized in different black rice cultivars [46]. As

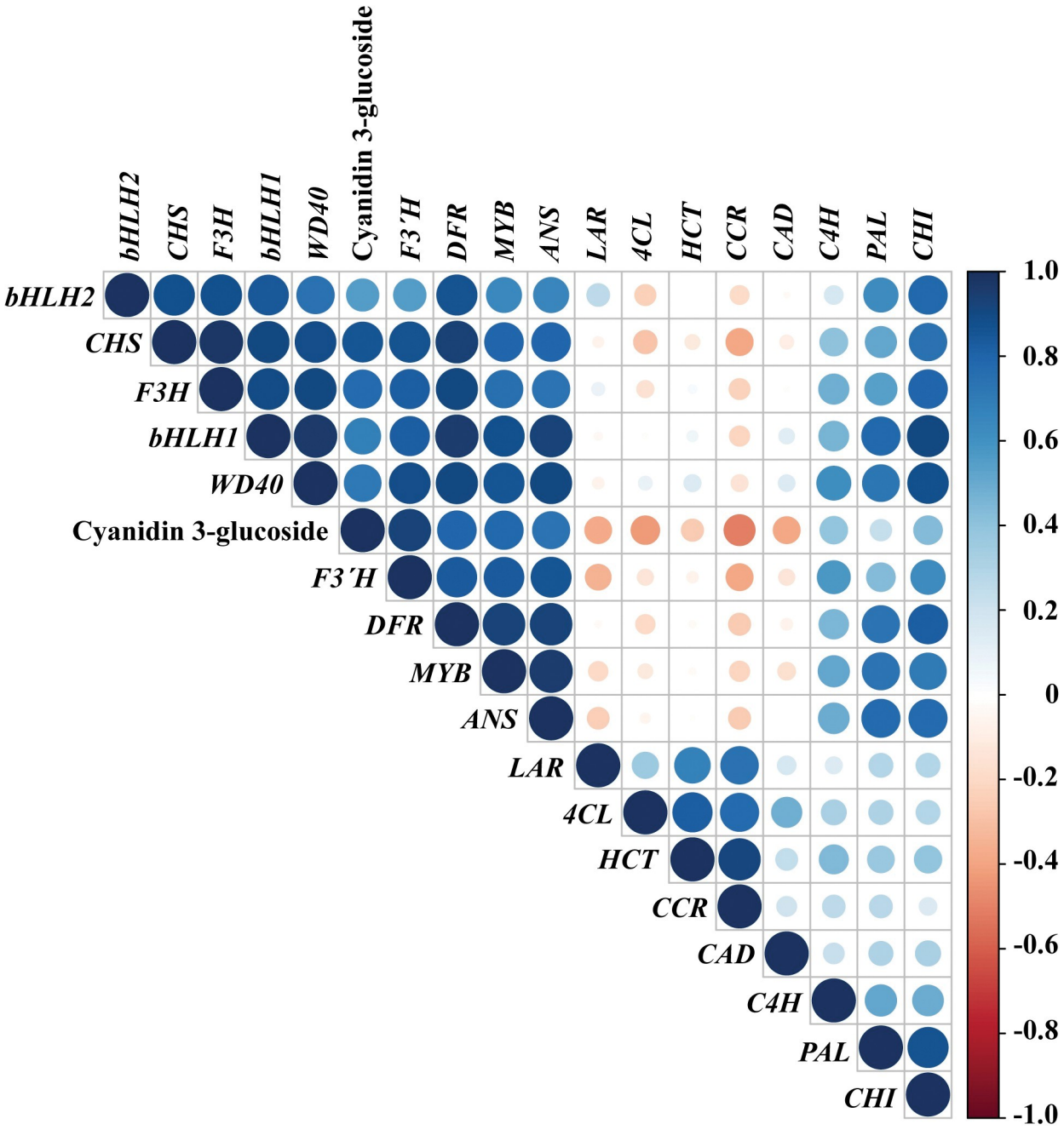

Fig 5. Correlation analysis in Dongjin, Geonganghongmi, Jeokjinju, Boseokheukchal, Heukjinju, Heukjinmi, and Heukseol, between the quantity of cyanidin 3-glucoside in hulled rice seeds and the expression level of each gene involved in the biosynthetic pathway of anthocyanins in seeds at twenty days after heading, and expression level of these genes. Bigger circles indicate more statistical significance. Blue and red colors show a positive and negative correlation, respectively.

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Kim et al. described [46], any detectable C3G was not identified in seeds of Dongjin, a white rice cultivar, and Geonganghongmi and Jeokjinju, red rice cultivars, and, in black rice, two groups showed statistically different quantities of C3G (Fig 2).

As reported in maize, the biosynthesis of anthocyanins was regulated by two transcription factors: *R*, a *bHLH* gene, and *C1*, a myb gene [16–19]. The overexpression of *C1* and *B-Peru*, a

bHLH gene, resulted in the biosynthesis of anthocyanins in developing white rice seeds [21]. Furthermore, the overexpression of maize *C1* and rice *bHLH* gene [*OSB1* (AB021079, *LOC_Os04g47080*) or *OSB2* (AB021080, *LOC_Os04g47059*)] resulted in the accumulation of anthocyanins in developing white rice seeds. However, no anthocyanin accumulation occurred in developing rice seeds upon the overexpression of only one gene of *C1*, *B-Peru*, *OSB1*, or *OSB2*. These results suggested that, in developing rice seeds, the overexpression of *R*, *bHLH* gene, and *C1*, myb gene, is essential for the biosynthesis of anthocyanins [21]. The *Kala4* (*LOC_Os04g47059*), an essential *bHLH* gene involved in the biosynthesis of anthocyanins in rice seeds, was upregulated in developing rice seeds more than in those white rice seeds [45]. The overexpression of *Kala4* led to the accumulation of anthocyanins in near-isogenic rice lines with *Kala3*, a myb gene functionally expressed and without *Kala4* being functionally expressed [45, 67]. In our RNA-seq data at 20 DAH rice seeds, we identified fifty-four *bHLH*s and seventy-five *MYB*s, and, of them, three *bHLH*s, including *LOC_Os01g09990*, *LOC_Os04g47040* (*bHLH2*), and *LOC_Os04g47059* (*bHLH1*; known as *OSB2* or *Kala4*), and 3 *MYB*s, including *LOC_Os01g49160* (*MYB*), *LOC_Os01g74410*, and *LOC_Os03g29614*, were differentially upregulated in developing black rice seeds. This indicated that those genes are putatively involved in the biosynthesis of anthocyanins in developing seeds of black rice (S5 Table). Interestingly, the *MYB* gene (Y15219, *LOC_Os06g10350*) homologous to maize *C1* reported by Reddy et al. did not show black rice-specific expression patterns in developing seeds [20], thereby indicating that there are other putative functional *MYB*s, involved in the biosynthesis of anthocyanins, with seed-specific expression patterns (S5 Table).

In addition, *WD40* (*LOC_Os02g45810*) was reported to regulate the biosynthesis of anthocyanins in *Arabidopsis thaliana* [59] and rice [68] and was differentially upregulated in black rice seeds (Fig 3B). However, in contrast to *bHLH* and *MYB*, *WD40* might be functionally expressed in developing white rice seeds because Sakamoto et al. showed the accumulation of anthocyanins in seeds of white rice by the overexpression of *bHLH* and *MYB*, but not with WD40 [21].

The structural genes, including *CHS* (*LOC_Os11g32650*), *F3H* (*LOC_Os04g56700*), *F3′H* (*LOC_Os10g17260*), *DFR* (*LOC_Os01g44260*), and *ANS* (*LOC_Os01g27490*), involved in the biosynthetic pathway of anthocyanins, were differentially upregulated in developing black rice seeds (S1 Fig), compared to the expression data in Nipponbare, a white rice cultivar, with no such upregulation as mentioned above (S2 Fig). Further verification of RNA-seq data by qRT-PCR exhibited that, of the structural genes in the biosynthetic pathway of anthocyanins, *CHS* (*LOC_Os11g32650*), *F3H* (*LOC_Os04g56700*), *F3′H* (*LOC_Os10g17260*), and *DFR* (*LOC_Os01g44260*) were differentially upregulated in developing seeds of black rice (Fig 3C). Moreover, *ANS* (*LOC_Os01g27490*), which converts leucoanthocyanidin into anthocyanidin [39–41], was expressed only in developing seeds of black rice but not in seeds of white and red rice (Fig 4A and S5 Table). However, there are remaining questions about the switch-on system for expressing *ANS* in black rice seeds, and it is required to carry out further investigations for this issue.

Catechin is converted from leucocynidin, a precursor shared by *LAR* [64] and *ANS* [39–41], by the reaction of *LAR* [64], and procyanidins were polymerized from catechin [64]. They were biosynthesized in seeds of red rice but not in white and black rice seeds [66]. As mentioned above, the expression of *LAR* (*LOC_Os03g15360*) was significantly upregulated in the seeds of Heukjinmi, which was crossed with red rice, as in the seeds of red rice cultivars, Geonganghongmi and Jeokjinju (Fig 4B). However, it was also significantly upregulated in the seeds of Boseokheukchal, compared to Dognjin (white rice) and the other two black rice cultivars, Heukjinju and Heukseol, indicating that higher expression of *LAR* is putatively related to the reduction of C3G biosynthesized in black rice seeds (Figs 2 and 4B). For Boseokheukchal, it is

necessary to investigate whether red rice is incorporated as a parent. These expression analysis data are closely related to the C3G quantities in four black rice cultivars, divided into the first group of Heukjinju and Heukseol with significantly higher C3G content, and the second group of Boseokheukchal and Heukjinmi with significantly lower C3G content (Fig 2).

Furthermore, correlation analysis between C3G contents in hulled rice and the expression level of genes involved in the biosynthesis of anthocyanins revealed that the quantities of anthocyanins biosynthesized in black rice seeds are positively correlated to the expression level of *bHLH1* (*LOC_Os04g47059*), *bHLH2* (*LOC_Os04g47040*), *MYB* (*LOC_Os01g49160*), *WD40* (*LOC_Os02g45810*), *CHS* (*LOC_Os11g32650*), *F3H* (*LOC_Os04g56700*), *F3´H* (*LOC_Os10g17260*), *DFR* (*LOC_Os01g44260*), and *ANS* (*LOC_Os01g27490*), respectively (Fig 5 and S6 Table). However, there is still little doubt about the biosynthesis of anthocyanins in rice seeds because we carried out this study with limited information and just a few black rice cultivars. Therefore, with more black rice cultivars and with much deeper details of transcriptomic and genomic data, further studies are required to perfectly understand the biosynthesis of anthocyanins in developing seeds of black rice, thereby will resulting in establishment of database for gene expression of each gene related to the biosynthetic pathway of anthocyanins in developing seeds in various black rice cultivars. Furthermore, the results from further studies can efficiently and powerfully be utilized in rice breeding programs to improve the anthocyanin content in seeds. In addition, as shown in Figs 3 and 4, red rice cultivars exhibited very unique expression data, compared to those in white and black rice cultivars. Further study is also needed for more understanding of the biosynthetic pathway of proanthocyanidins in developing seeds of red rice through transcriptomic and genomic analysis tools.

Conclusion

In this study, we elucidated that the C3G contents biosynthesized in black rice seeds positively correlate to the expression levels of genes, including *bHLH1*, *bHLH2*, *MYB*, *WD40*, *CHS*, *F3H*, *F3´H*, *DFR*, and *ANS*. In addition, compared to those of white and red rice cultivars, several genes that regulate the biosynthesis of anthocyanins, including *bHLH*s, *MYB*s, and *WD40*, were highly upregulated in developing seeds of black rice cultivars, and the structural genes in the biosynthetic pathway of anthocyanins, including *CHS*, *F3H*, *F3´H*, *DFR*, and *ANS*, were also differentially upregulated in black rice seeds. Moreover, we report new candidate transcription factor genes, *bHLH2* (*LOC_Os04g47040*), and *MYB*s (*LOC_Os01g49160*, *LOC_Os01g74410*, and *LOC_Os03g29614*), with black rice seed-specific expression patterns, for the biosynthesis of anthocyanins in black rice seeds.

Supporting information

S1 Fig. The relative expression of genes involved in the phenylpropanoid pathway and biosynthetic pathway of anthocyanins, detected in the seeds of Dongjin, Jeokjinju, and Heukseol at twenty days after heading through RNA-seq. *bHLH*: basic helix-loop-helix gene; *MYB*: myb gene; *WD40*: tryptophan-aspartic acid repeat protein gene; *PAL*: *phenylalanine ammonia-lyase*; *C4H*: *cinnamate 4-hydroxylase*; *4CL*: *4-coumarate*: *CoA ligase*; *HCT*: *hydroxycinnamoyl-CoA shikimate/quinate hydroxycinnamoyltransferase*; *CCR*: *cinnamoyl-CoA reductase*; *CAD*: *cinnamyl alcohol dehydrogenase*; *CHS*: *chalcone synthase*; *CHI*: *chalcone isomerase*; *F3H*: *flavanone 3-hydroxylase*; *DFR*: *dihydroflavonol 4-reductase*; *F3´H*: *flavonoid 3´-hydroxylase*; *ANS*: *anthocyanidin synthase*; and *LAR*: *leucoanthocyanidin reductase*. The scale bar indicates the normalized Log_2 ratio (individual value/average value).
(TIF)

S2 Fig. The relative expression of genes, involved in the phenylpropanoid pathway and biosynthetic pathway of anthocyanins, detected in Nipponbare (white rice) obtained from the RNA-seq data of the Rice Genome Annotation Project (http://rice.plantbiology.msu.edu/). *: days after pollination. The scale bar indicates the normalized Log2 ratio (individual value/average value).
(TIF)

S3 Fig. The relative expression of genes involved in the phenylpropanoid pathway in developing seeds of Dongjin, Geonganghongmi, Jeokjinju, Boseokheukchal, Heukjinju, Heukjinmi, and Heukseol at twenty days after heading through a quantitative real-time polymerase chain reaction. A. *PAL* (*LOC_Os02g41630*), *C4H* (*LOC_Os05g25640*), and *4CL* (*LOC_Os02g08100*); **B.** *HCT* (*LOC_Os04g42250*), *CCR* (*LOC_Os09g25150*), and *CAD* (*LOC_Os02g09490*). The data represents mean ± standard deviation (SD). #, ¶, ^: Duncan's Multiple Range Test (DMRT, $\alpha = 0.05$) was performed after confirming statistical significance for these data through analysis of variance (ANOVA).
(TIF)

S4 Fig. The relative expression of *CHI* (*LOC_Os03g60509*) in the seeds of Dongjin, Geonganghongmi, Jeokjinju, Boseokheukchal, Heukjinju, Heukjinmi, and Heukseol at twenty days after heading through a quantitative real-time polymerase chain reaction. The data represents mean ± standard deviation (SD). #: Duncan's Multiple Range Test (DMRT, $\alpha = 0.05$) was performed after the confirmation of statistical significance for these data through analysis of variance (ANOVA).
(TIF)

S1 Table. Basic information of raw data generated from RNA sequencing.
(DOCX)

S2 Table. The expression value of genes detected in seeds of Dongjin, Jeokjinju, and Heukseol, respectively, at twenty days after heading through RNA-seq analysis. (expression values of each gene was normalized by division of the expression values of *OsUBI1* of Dongjin, Jeokjinju, or Heukseol, respectively).
(XLSX)

S3 Table. List of primer sets for a quantitative real-time polymerase chain reaction.
(DOCX)

S4 Table. Black rice cultivars developed at the National Institute of Crop Science, Republic of Korea. Retrieved from: http://www.nics.go.kr/api/breed.do?m=100000128&homepageSeCode=nics.
(DOCX)

S5 Table. The genes, putatively involved in the biosynthetic pathway of anthocyanins, selected from RNA-seq data of seeds of Dongjin, Jeokjinju, and Heukseol, respectively, at twenty days after heading. (expression values of each gene was normalized by division of the expression values of *OsUBI1* of Dongjin, Jeokjinju, or Heukseol, respectively).
(XLSX)

S6 Table. The correlation coefficient, in Dongjin, Geonganghongmi, Jeokjinju, Boseokheukchal, Heukjinju, Heukjinmi, and Heukseol, obtained from correlation analysis between the quantity of cyanidin 3-glucoside in hulled rice seeds and the expression levels of each gene involved in the biosynthetic pathway of anthocyanins in seeds at twenty days

after heading and between the expression levels of these genes.
(DOCX)

Author Contributions

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Methodology: Choonseok Lee.

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Supervision: Hee-Jong Koh, Ki-Hong Jung.

Validation: Choonseok Lee.

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Writing – original draft: Choonseok Lee, Yang-Seok Lee, Woo-Jong Hong, Hee-Jong Koh, Ki-Hong Jung.

References

1. Iwashina T (2000) The Structure and Distribution of the Flavonoids in Plants. Journal of Plant Research 113: 287–299. https://doi.org/10.1007/PL00013940
2. Scott-Moncrieff R (1930) Natural anthocyanin pigments: The magenta flower pigment of Primula polyanthus. The Biochemical journal 24: 767–778. https://doi.org/10.1042/bj0240767 PMID: 16744417
3. Grove KE, Robinson R (1931) An anthocyanin of Oxycoccus macrocarpus Pers. The Biochemical journal 25: 1706–1711. https://doi.org/10.1042/bj0251706 PMID: 16744736
4. Sondheimer E, Kertesz ZI (1948) The Anthocyanin of Strawberries1. Journal of the American Chemical Society 70: 3476–3479. https://doi.org/10.1021/ja01190a079 PMID: 18891898
5. Koukol J, Dugger WM Jr., (1967) Anthocyanin formation as a response to ozone and smog treatment in Rumex crispus L. Plant Physiology 42: 1023–1024. https://doi.org/10.1104/pp.42.7.1023 PMID: 6047104
6. McClure JW (1968) Photocontrol of Spirodela intermedia flavonoids. Plant Physiology 43: 193–200. https://doi.org/10.1104/pp.43.2.193 PMID: 16656751
7. Gupta SB (1970) Biochemical aspects of the inheritance of floral anthocyanins in diploid alfalfa. Genetics 65: 267–278. https://doi.org/10.1093/genetics/65.2.267 PMID: 17248498
8. Reddy VS, Dash S, Reddy AR (1995) Anthocyanin pathway in rice (Oryza sativa L): identification of a mutant showing dominant inhibition of anthocyanins in leaf and accumulation of proanthocyanidins in pericarp. Theoretical and Applied Genetics 91: 301–312. https://doi.org/10.1007/BF00220892 PMID: 24169778
9. Min B, McClung AM, Chen M-H (2011) Phytochemicals and Antioxidant Capacities in Rice Brans of Different Color. Journal of Food Science 76: C117–C126. https://doi.org/10.1111/j.1750-3841.2010.01929.x PMID: 21535639
10. Yoshimura Y, Zaima N, Moriyama T, Kawamura Y (2012) Different localization patterns of anthocyanin species in the pericarp of black rice revealed by imaging mass spectrometry. PLoS ONE 7: e31285–e31285. https://doi.org/10.1371/journal.pone.0031285 PMID: 22363605
11. Lee S, Stenger DC, Bisaro DM, Davies KR (1994) Identification of loci in Arabidopsis that confer resistance to geminivirus infection. The Plant Journal 6: 525–535. https://doi.org/10.1046/j.1365-313x.1994.6040525.x PMID: 7987411

12. Lindoo SJ, Caldwell MM (1978) Ultraviolet-B Radiation-induced Inhibition of Leaf Expansion and Promotion of Anthocyanin Production: Lack of Involvement of the Low Irradiance Phytochrome System. Plant Physiology 61: 278–282. https://doi.org/10.1104/pp.61.2.278 PMID: 16660276

13. Reddy VS, Goud KV, Sharma R, Reddy AR (1994) Ultraviolet-B-Responsive Anthocyanin Production in a Rice Cultivar Is Associated with a Specific Phase of Phenylalanine Ammonia Lyase Biosynthesis. Plant Physiology 105: 1059–1066. https://doi.org/10.1104/pp.105.4.1059 PMID: 12232265

14. Tsuda T, Shiga K, Ohshima K, Kawakishi S, Osawa T (1996) Inhibition of lipid peroxidation and the active oxygen radical scavenging effect of anthocyanin pigments isolated from Phaseolus vulgaris L. Biochemical Pharmacology 52: 1033–1039. https://doi.org/10.1016/0006-2952(96)00421-2 PMID: 8831722

15. Wang H, Nair MG, Strasburg GM, Chang Y-C, Booren AM, et al. (1999) Antioxidant and Antiinflammatory Activities of Anthocyanins and Their Aglycon, Cyanidin, from Tart Cherries. Journal of Natural Products 62: 294–296. https://doi.org/10.1021/np980501m PMID: 10075763

16. Paz-Ares J, Wienand U, Peterson PA, Saedler H (1986) Molecular cloning of the c locus of Zea mays: a locus regulating the anthocyanin pathway. The EMBO Journal 5: 829–833. https://doi.org/10.1002/j.1460-2075.1986.tb04291.x PMID: 15957214

17. Paz-Ares J, Ghosal D, Wienand U, Peterson PA, Saedler H (1987) The regulatory c1 locus of Zea mays encodes a protein with homology to myb proto-oncogene products and with structural similarities to transcriptional activators. The EMBO Journal 6: 3553–3558. https://doi.org/10.1002/j.1460-2075.1987.tb02684.x PMID: 3428265

18. Ludwig SR, Habera LF, Dellaporta SL, Wessler SR (1989) Lc, a member of the maize R gene family responsible for tissue-specific anthocyanin production, encodes a protein similar to transcriptional activators and contains the myc-homology region. Proceedings of the National Academy of Sciences of the United States of America 86: 7092–7096. https://doi.org/10.1073/pnas.86.18.7092 PMID: 2674946

19. Lloyd AM, Walbot V, Davis RW (1992) *Arabidopsis* and *Nicotiana* Anthocyanin Production Activated by Maize Regulators *R* and *C1*. Science 258: 1773–1775. https://doi.org/10.1126/science.1465611 PMID: 1465611

20. Reddy VS, Scheffler BE, Wienand U, Wessler SR, Reddy AR (1998) Sequence Annoucement. Plant Molecular Biology 36: 497–498. https://doi.org/10.1023/a:1017106913186

21. Sakamoto W, Ohmori T, Kageyama K, Miyazaki C, Saito A, et al. (2001) The Purple leaf (Pl) Locus of Rice: the Plw Allele has a Complex Organization and Includes Two Genes Encoding Basic Helix-Loop-Helix Proteins Involved in Anthocyanin Biosynthesis. Plant and Cell Physiology 42: 982–991. https://doi.org/10.1093/pcp/pce128 PMID: 11577193

22. Appert C, Logemann E, Hahlbrock K, Schmid J, Amrhein N (1994) Structural and Catalytic Properties of the Four Phenylalanine Ammonia-Lyase Isoenzymes from Parsley (Petroselinum Crispum Nym.). European Journal of Biochemistry 225: 491–499. https://doi.org/10.1111/j.1432-1033.1994.00491.x PMID: 7925471

23. Teutsch HG, Hasenfratz MP, Lesot A, Stoltz C, Garnier JM, et al. (1993) Isolation and sequence of a cDNA encoding the Jerusalem artichoke cinnamate 4-hydroxylase, a major plant cytochrome P450 involved in the general phenylpropanoid pathway. Proceedings of the National Academy of Sciences of the United States of America 90: 4102–4106. https://doi.org/10.1073/pnas.90.9.4102 PMID: 8097885

24. URBAN P, WERCK-REICHHART D, TEUTSCH HG, DURST F, REGNIER S, et al. (1994) Characterization of recombinant plant cinnamate 4-hydroxylase produced in yeast. European Journal of Biochemistry 222: 843–850. https://doi.org/10.1111/j.1432-1033.1994.tb18931.x PMID: 8026495

25. LOZOYA E, HOFFMANN H, DOUGLAS C, SCHULZ W, SCHEEL D, et al. (1988) Primary structures and catalytic properties of isoenzymes encoded by the two 4-coumarate: CoA ligase genes in parsley. European Journal of Biochemistry 176: 661–667. https://doi.org/10.1111/j.1432-1033.1988.tb14328.x PMID: 3169018

26. Heller W, Hahlbrock K (1980) Highly purified "flavanone synthase" from parsley catalyzes the formation of naringenin chalcone. Archives of Biochemistry and Biophysics 200: 617–619. https://doi.org/10.1016/0003-9861(80)90395-1 PMID: 7436427

27. Nakajima O, Akiyama T, Hakamatsuka T, Shibuya M, Noguchi H, et al. (1991) ISOLATION, SEQUENCE AND BACTERIAL EXPRESSION OF A cDNA FOR CHALCONE SYNTHASE FROM THE CULTURED CELLS OF PUERARIA LOBATA. CHEMICAL & PHARMACEUTICAL BULLETIN 39: 1911–1913. https://doi.org/10.1248/cpb.39.1911 PMID: 1777944

28. Forkmann G, Kuhn B (1979) Genetic control of chalcone isomerase activity in anthers of Petunia hybrida. Planta 144: 189–192. https://doi.org/10.1007/BF00387269 PMID: 24408692

29. van Tunen AJ, Koes RE, Spelt CE, van der Krol AR, Stuitje AR, et al. (1988) Cloning of the two chalcone flavanone isomerase genes from Petunia hybrida: coordinate, light-regulated and differential

expression of flavonoid genes. The EMBO Journal 7: 1257–1263. https://doi.org/10.1002/j.1460-2075.1988.tb02939.x PMID: 3409864

30. Bednar RA, Hadcock JR (1988) Purification and characterization of chalcone isomerase from soybeans. Journal of Biological Chemistry 263: 9582–9588. https://doi.org/10.1016/s0021-9258(19)81556-9 PMID: 3384815

31. BRITSCH L, GRISEBACH H (1986) Purification and characterization of (2S)-flavanone 3-hydroxylase from Petunia hybrida. European Journal of Biochemistry 156: 569–577. https://doi.org/10.1111/j.1432-1033.1986.tb09616.x PMID: 3699024

32. Britsch L, Ruhnau-Brich B, Forkmann G (1992) Molecular cloning, sequence analysis, and in vitro expression of flavanone 3 beta-hydroxylase from Petunia hybrida. Journal of Biological Chemistry 267: 5380–5387. https://doi.org/10.1016/S0021-9258(18)42777-9 PMID: 1544919

33. Fischer D, Stich K, Britsch L, Grisebach H (1988) Purification and characterization of (+)dihydroflavonol (3-hydroxyflavanone) 4-reductase from flowers of Dahlia variabilis. Archives of Biochemistry and Biophysics 264: 40–47. https://doi.org/10.1016/0003-9861(88)90567-x PMID: 3293532

34. Helariutta Y, Elomaa P, Kotilainen M, Seppänen P, Teeri TH (1993) Cloning of cDNA coding for dihydroflavonol-4-reductase (DFR) and characterization of dfr expression in the corollas of Gerbera hybrida var. Regina (Compositae). Plant Molecular Biology 22: 183–193. https://doi.org/10.1007/BF00014927 PMID: 8507822

35. Tanaka Y, Fukui Y, Fukuchi-Mizutani M, Holton TA, Higgins E, et al. (1995) Molecular Cloning and Characterization of Rosa hybrida Dihydroflavonol 4-reductase Gene. Plant and Cell Physiology 36: 1023–1031. https://doi.org/10.1093/oxfordjournals.pcp.a078844 PMID: 8528604

36. HAGMANN M-L, HELLER W, GRISEBACH H (1983) Induction and Characterization of a Microsomal Flavonoid 3′-Hydroxylase from Parsley Cell Cultures. European Journal of Biochemistry 134: 547–554. https://doi.org/10.1111/j.1432-1033.1983.tb07601.x PMID: 6884346

37. Brugliera F, Barri-Rewell G, Holton TA, Mason JG (1999) Isolation and characterization of a flavonoid 3′-hydroxylase cDNA clone corresponding to the Ht1 locus of Petunia hybrida. The Plant Journal 19: 441–451. https://doi.org/10.1046/j.1365-313x.1999.00539.x PMID: 10504566

38. Holton TA, Brugliera F, Lester DR, Tanaka Y, Hyland CD, et al. (1993) Cloning and expression of cytochrome P450 genes controlling flower colour. Nature 366: 276–279. https://doi.org/10.1038/366276a0 PMID: 8232589

39. Menssen A, Höhmann S, Martin W, Schnable PS, Peterson PA, et al. (1990) The En/Spm transposable element of Zea mays contains splice sites at the termini generating a novel intron from a dSpm element in the A2 gene. The EMBO Journal 9: 3051–3057. https://doi.org/10.1002/j.1460-2075.1990.tb07501.x PMID: 2170105

40. Nakajima J-i, Tanaka Y, Yamazaki M, Saito K (2001) Reaction Mechanism from Leucoanthocyanidin to Anthocyanidin 3-Glucoside, a Key Reaction for Coloring in Anthocyanin Biosynthesis. Journal of Biological Chemistry 276: 25797–25803. https://doi.org/10.1074/jbc.M100744200 PMID: 11316805

41. Reddy AM, Reddy VS, Scheffler BE, Wienand U, Reddy AR (2007) Novel transgenic rice overexpressing anthocyanidin synthase accumulates a mixture of flavonoids leading to an increased antioxidant potential. Metabolic Engineering 9: 95–111. https://doi.org/10.1016/j.ymben.2006.09.003 PMID: 17157544

42. Ford CM, Boss PK, Høj PB (1998) Cloning and Characterization of Vitis viniferaUDP-Glucose:Flavonoid 3-O-Glucosyltransferase, a Homologue of the Enzyme Encoded by the Maize Bronze-1Locus That May Primarily Serve to Glucosylate Anthocyanidins in Vivo. Journal of Biological Chemistry 273: 9224–9233. https://doi.org/10.1074/jbc.273.15.9224 PMID: 9535914

43. Yoshihara N, Imayama T, Fukuchi-Mizutani M, Okuhara H, Tanaka Y, et al. (2005) cDNA cloning and characterization of UDP-glucose: Anthocyanidin 3-O-glucosyltransferase in Iris hollandica. Plant Science 169: 496–501. https://doi.org/10.1016/j.plantsci.2005.04.007

44. Kim BG, Kim JH, Min SY, Shin K-H, Kim JH, et al. (2007) Anthocyanin content in rice is related to expression levels of anthocyanin biosynthetic genes. Journal of Plant Biology 50: 156–160. https://doi.org/10.1007/BF03030624

45. Oikawa T, Maeda H, Oguchi T, Yamaguchi T, Tanabe N, et al. (2015) The Birth of a Black Rice Gene and Its Local Spread by Introgression. The Plant Cell 27: 2401–2414. https://doi.org/10.1105/tpc.15.00310 PMID: 26362607

46. Kim JK, Lee SY, Chu SM, Lim SH, Suh S-C, et al. (2010) Variation and Correlation Analysis of Flavonoids and Carotenoids in Korean Pigmented Rice (Oryza sativa L.) Cultivars. Journal of Agricultural and Food Chemistry 58: 12804–12809. https://doi.org/10.1021/jf103277g PMID: 21090621

47. Cho E, Chung YE, Jang H-Y, Hong O-Y, Chae SH, et al. (2017) Anti-cancer Effect of Cyanidin-3-glucoside from Mulberry via Caspase-3 Cleavage and DNA Fragmentation in vitro and in vivo. Anti-Cancer

Agents in Medicinal Chemistry 17: 1519–1525. https://doi.org/10.2174/1871520617666170327152026 PMID: 28356020

48. Lee C, Hong W-J, Jung K-H, Hong H-C, Kim D-Y, et al. (2021) Arachis hypogaea resveratrol synthase 3 alters the expression pattern of UDP-glycosyltransferase genes in developing rice seeds. PLoS ONE 16: e0245446–e0245446. https://doi.org/10.1371/journal.pone.0245446 PMID: 33444365

49. Martin M (2011) Cutadapt removes adapter sequences from high-throughput sequencing reads. EMBnetjournal 17: 10–12. https://doi.org/10.14806/ej.17.1.200

50. Pertea M, Kim D, Pertea GM, Leek JT, Salzberg SL (2016) Transcript-level expression analysis of RNA-seq experiments with HISAT, StringTie and Ballgown. Nature Protocols 11: 1650–1667. https://doi.org/10.1038/nprot.2016.095 PMID: 27560171

51. Liao Y, Smyth GK, Shi W (2014) featureCounts: an efficient general purpose program for assigning sequence reads to genomic features. Bioinformatics 30: 923–930. https://doi.org/10.1093/bioinformatics/btt656 PMID: 24227677

52. Lee Y-S, Lee D-Y, Cho L-H, An G (2014) Rice miR172 induces flowering by suppressing OsIDS1 and SNB, two AP2 genes that negatively regulate expression of Ehd1 and florigens. Rice 7: 31. https://doi.org/10.1186/s12284-014-0031-4 PMID: 26224560

53. Rahman MM, Rahman MM, Eom J-S, Jeon J-S (2021) Genome-wide Identification, Expression Profiling and Promoter Analysis of Trehalose-6-Phosphate Phosphatase Gene Family in Rice. Journal of Plant Biology 64: 55–71. https://doi.org/10.1007/s12374-020-09279-x

54. Kumar V, Kim SH, Adnan MR, Heo J, Jeong JH, et al. (2021) Tiller Outgrowth in Rice (Oryza sativa L.) is Controlled by OsGT1, Which Acts Downstream of FC1 in a PhyB-Independent Manner. Journal of Plant Biology 64: 417–430. https://doi.org/10.1007/s12374-021-09310-9

55. Pfaffl MW (2004) Quantification strategies in real-time PCR. In: Bustin SA, editor. A-Z of quantitative PCR. La Jolla, CA. U.S.A.: International University Line (IUL). pp. 87–112.

56. Haarman BCM, Riemersma-Van der Lek RF, Nolen WA, Mendes R, Drexhage HA, et al. (2015) Feature-expression heat maps–A new visual method to explore complex associations between two variable sets. Journal of Biomedical Informatics 53: 156–161. https://doi.org/10.1016/j.jbi.2014.10.003 PMID: 25445923

57. Rahman MM, Lee KE, Kang SG (2016) Allelic Gene Interaction and Anthocyanin Biosynthesis of *Purple Pericarp* Trait for Yield Improvement in Black Rice. Journal of Life Science 26: 727–736. https://doi.org/10.5352/JLS.2016.26.6.727

58. Mikami I, Takahashi A, Khin-Thidar, Sano Y(2000) A candidate for *C* (*Chromogen for anthocyanin*) gene. Rice Genetics Newsletter 17: 54–55.

59. Walker AR, Davison PA, Bolognesi-Winfield AC, James CM, Srinivasan N, et al. (1999) The *TRANSPARENT TESTA GLABRA1* Locus, Which Regulates Trichome Differentiation and Anthocyanin Biosynthesis in Arabidopsis, Encodes a WD40 Repeat Protein. The Plant Cell 11: 1337–1349. https://doi.org/10.1105/tpc.11.7.1337 PMID: 10402433

60. Hoffmann L, Maury S, Martz F, Geoffroy P, Legrand M (2003) Purification, Cloning, and Properties of an Acyltransferase Controlling Shikimate and Quinate Ester Intermediates in Phenylpropanoid Metabolism*. Journal of Biological Chemistry 278: 95–103. https://doi.org/10.1074/jbc.M209362200 PMID: 12381722

61. Goffner D, Campbell MM, Campargue C, Clastre M, Borderies G, et al. (1994) Purification and Characterization of Cinnamoyl-Coenzyme A:NADP Oxidoreductase in Eucalyptus gunnii. Plant Physiology 106: 625–632. https://doi.org/10.1104/pp.106.2.625 PMID: 12232355

62. Lacombe E, Hawkins S, Van Doorsselaere J, Piquemal J, Goffner D, et al. (1997) Cinnamoyl CoA reductase, the first committed enzyme of the lignin branch biosynthetic pathway: cloning, expression and phylogenetic relationships. The Plant Journal 11: 429–441. https://doi.org/10.1046/j.1365-313x.1997.11030429.x PMID: 9107033

63. Kim S-J, Kim M-R, Bedgar DL, Moinuddin SGA, Cardenas CL, et al. (2004) Functional reclassification of the putative cinnamyl alcohol dehydrogenase multigene family in *Arabidopsis*. Proceedings of the National Academy of Sciences of the United States of America 101: 1455–1460. https://doi.org/10.1073/pnas.0307987100 PMID: 14745009

64. Tanner GJ, Francki KT, Abrahams S, Watson JM, Larkin PJ, et al. (2003) Proanthocyanidin Biosynthesis in Plants: PURIFICATION OF LEGUME LEUCOANTHOCYANIDIN REDUCTASE AND MOLECULAR CLONING OF ITS cDNA *. Journal of Biological Chemistry 278: 31647–31656. https://doi.org/10.1074/jbc.M302783200 PMID: 12788945

65. Anterola AM, Lewis NG (2002) Trends in lignin modification: a comprehensive analysis of the effects of genetic manipulations/mutations on lignification and vascular integrity. Phytochemistry 61: 221–294. https://doi.org/10.1016/s0031-9422(02)00211-x PMID: 12359514

66. Zaupa M, Calani L, Del Rio D, Brighenti F, Pellegrini N (2015) Characterization of total antioxidant capacity and (poly)phenolic compounds of differently pigmented rice varieties and their changes during domestic cooking. Food Chemistry 187: 338–347. https://doi.org/10.1016/j.foodchem.2015.04.055 PMID: 25977035

67. Maeda H, Yamaguchi T, Omoteno M, Takarada T, Fujita K, et al. (2014) Genetic dissection of black grain rice by the development of a near isogenic line. Breeding Science 64: 134–141. https://doi.org/10.1270/jsbbs.64.134 PMID: 24987299

68. Yang X, Wang J, Xia X, Zhang Z, He J, et al. (2021) OsTTG1, a WD40 repeat gene, regulates anthocyanin biosynthesis in rice. The Plant Journal 107: 198–214. https://doi.org/10.1111/tpj.15285 PMID: 33884679