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Research Article

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Differential expression profiles of anthocyanidin biosynthesis gene during black rice seed development

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ABSTRACT

The black rice (*Oryza sativa* cv. Heugjinju) is rich in anthocyanins which is beneficial to human health. To correlate the biosynthesis of the pigments with relevant genes, the mRNA level of genes involved in anthocyanin biosynthesis was monitored by quantitative real-time polymerase chain reaction (qRT-PCR) during seed development of black rice. The mRNA level of F3'H, DFR, and ANS, key enzymes in anthocyanidin biosynthesis, peaked at 10 days after flowering. In general, the absolute level of ANS was approximately one-fold higher than F3'H, F3'5'H, and DFR in 10 days after flowering. The transcript level of major seed protein gene GluA-3, taken as reference, was also at the highest on the 10 days after flowering. However, the level of CHS isogenes was highest at 15 or 20 days after flowering. The highest transcript level of the genes, except CHS, preceded the highest anthocyanidin content by 5 days. This pattern coincided with an increase of anthocyanin content between 10 and 15 days after flowering. From these findings, it is suggested that particular CHS isoforms might be responsible for the anthocyanin production in black rice.

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INTRODUCTION

The anthocyanins belong to a widespread group of secondary metabolites collectively called flavonoids. They present in a flower petal, leaves, fruits, roots, and stem of higher plants and protect them from attracting the pollinators and animals for seed dispersal (Holton & Cornish, 1995). The anthocyanins also have other important roles in plant ecology (Gould, 2004). For example, cyanidin 3-glucoside protects cotton leaves against the tobacco budworm (Hedin *et al*., 1983). The anthocyanins also have beneficial activities on human health. They possess antidiabetic, antioxidant, antimicrobial, anti-inflammatory, anti-obesity, tumor-arresting activities and it lowers the risk of cardiovascular diseases (Kong *et al*., 2003; Nam *et al*., 2005; Xia *et al*., 2006). Recently, efforts are being made to engineer crops to attain higher anthocyanin levels for the enhanced nutraceutical value (Shirley, 1998; Reddy *et al*., 2007). Black rice, an important crop in Asia, contains anthocyanin's as the major pigment components (Saito *et al*., 1985). The major anthocyanin in the Heugjinju variety, the most widely cultivated black rice in Korea noted for its high anthocyanin content, especially cyanidin 3-glucoside (Choi *et al*., 1996; Lee *et al*., 1998).

Anthocyanin biosynthesis involves several genes in the flavonoid biosynthetic pathway. Chalcone synthase (CHS), catalyzing the initial step in the flavonoid biosynthetic pathway, is highly regulated by the presence of multiple isozymes. Rice in particular contains a family of CHS composed of 30 members. Chalcone is converted by chalcone isomerase into naringenin, which is subsequently transformed into dihydrokaempferol (DHK) by flavanone 3-hydroxylase (F3'H), this enzyme acts on the B ring of DHK to result in the formation of dihydroquercetin, which is the precursor for the production of all cyanidin pigments (Fig. 1). DHK itself is the precursor of pelargonidin-type pigments (Forkmann, 1991). Additional hydroxylation on the B ring for blue delphinidine-based pigments requires the action of flavonol 3',5'-hydroxylase (F3'5'H) on DHK. Furthermore, the dihydroflavonols is catalyzed into leucoanthocyanidins by the enzyme dihydroflavonol 4-reductase (DFR). Dehydration of leucoanthocyanidin by the enzyme anthocyanidin synthase (ANS) is the final step for the production of anthocyanidins.

Glycosylation of the anthocyanidins, typically at C-3' of the ring B leads to the formation of various anthocyanins. The apparent color of the anthocyanidins in plant tissue is affected by various factors such as pH of the vacuoles, where anthocyanins are stored, complexation with metal ions, and conjugation with sugar moieties and organic acids (Strack & Wray, 1989).

The genes involved in the anthocyanin biosynthesis have been cloned and characterized from several plants (Holton & Cornish, 1995). However, the genes and enzymes responsible for anthocyanin biosynthesis in cereal crops have not yet been studied well (Lepiniec *et al.*, 2006). In particular, the anthocyanin-related genes in rice were not well-characterized even though the genomic sequence of the rice has been available. The main goal of the present research is to correlate the genes putatively involved in the anthocyanin biosynthesis with the pigment accumulation in black rice. In this regard, we studied the transcription level of the related genes in a time-course manner during seed maturation.

MATERIALS AND METHODS

Plant Materials

The rice cultivar (*Oryza sativa* cv. Heugjinju) was grown in the month of June-October, 2006 at the field of National Crop Experiment Station, Rural Development Administration, Suwon, Korea. The seeds were harvested from 5 to 40 days after flowering.

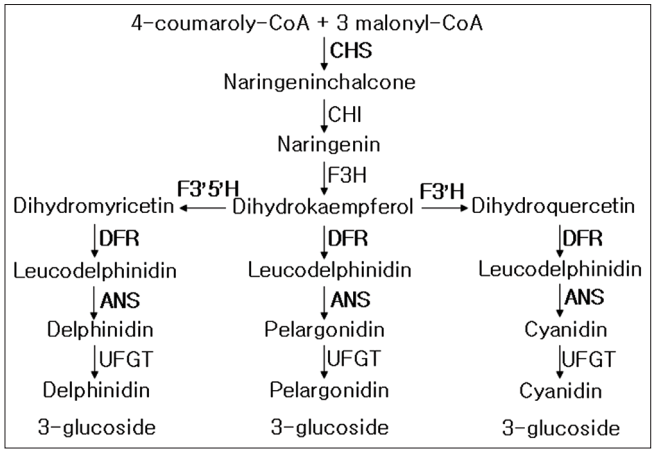

Figure 1: Simplified schematic of the anthocyanin biosynthetic pathway

Total RNA Extraction and cDNA Synthesis

Total RNA was isolated from the dehulled seeds using (Carpenter & Simon, 1998) protocol with minor modification in extraction buffer as follows: 200 mM Tris-HCl, 300 mM NaCl, 20 mM EDTA, 1% SDS, pH 8.5. Every chemical used in this study was purchased from Sigma-Aldrich St. Louis, MO, USA. Single-stranded cDNA was synthesized by the GeneRacer Kit (Invitrogen). Protocol for reverse transcriptase-polymerase chain reaction (RT-PCR) was done according to manufacture instructions (QiagenOmniscript). 1 µL of cDNA was made up to 20 µL volume and incubated for 60 min at 37°C and then at 95°C for 5 min.

Quantitative Real-time PCR and Anthocyanidin Content

The quantitative real-time polymerase chain reaction (qRT-PCR) primers were designed based on TIGR rice genome annotation (http://rice.plantbiology.msu.edu/cgi-bin/putative_function_search.pl) (Table 1). qRT-PCR was carried out using the Rotor-Gene 2000 Real-Time Amplification System (Corbett Research, Australia) and the qRT-PCR reaction mixture was carried out according to the manufacturer protocol (Quantitect SYBR Green PCR system, Qiagen). The thermal cycling conditions were as follows; stage 1, 95°C for 15 min; stage 2, 94°C for 15 s, 50°C (CHS1), 53°C (CHS2 and 3), 52°C (CHS4), 43°C (F3'H and F3'5'H) or 57°C (DFR) for 30 s, and stage 3, 72°C for 30 s. Stage 2 and 3 were repeated for 40 cycles. The reaction was run in triplicates. The standard curves for each gene, each ranging from 1 × 103 to 1 × 107 copy/µL, were prepared from PCR products of each gene as described by (Yin *et al.*, 2001).

Estimation of Anthocyanin Content

Anthocyanin compounds were extracted after removing the rice hull and analyzed following the protocol described by Park *et al.*, 2018.

RESULTS

Transcript Level of Anthocyanidin Biosynthesis Gene in Black Rice

There are 30 copies of CHS in the rice genome were identified (Fig. 2), among them 11 were differentially expressed. We

Table 1: Real-time PCR primers used in this study

Genes	Forward primer (5′ to 3′)	Reverse primer (5′ to 3′)
CHS1	CGGAGATCATCGACAAACACA	CAGCTTGAGGTCAGCGCTAGG
CHS2	CGTCACCACTGAGATCCCGA	GCTTGAGGTCAGCGCTTGGA
CHS3	GCATGTGCGTGTTCACGGC	ACTCCGTTGGTCGTGCAGAAC
CHS4	CACCCAGAGATCATTGACAAACATT	GTGCTGAAGATAAGGTGAGTGATGTC
CHS5	AAACCCGGCAAACATATGTCC	AGGCAATGTCTACACGAGATATTATC
CHS6	TCGCCAAGGACATCGCC	CGACCCCTGCCCCTAG
CHS7	AGCTCGAGAGGATCTATGTAGACAAG	CGATGCCGGATTTCTTACCTTG
F3′H	GATTCATCAACGAAAGGAAG	AGTGAATAGGTTCAGGAGCA
F3′5′H	AGCAAATCTTCTCTACGCTG	AAGTAGGTGTGCAAGTTTGG
DFR	GTCGCGACGCCCATGGACT	GCGCTGCCGCTCCTCGATGTTGACG
ANS	TCGTCGACATCTCCGCGTTC	ATGGGCAGCGCGAAGAACG
GluA-3	ATGGCAACCATCAAATTCCCTATAG	TTATGCACTCACAGATATGTCTTGGTAG

selected 23 CHSs longer than 1 kb so that all the known expressed sequences are included. Os12q07690 was excluded though it is known to be expressed because is too short to be a functional CHS. The selected genes were aligned and classified into 7 groups based on base sequence similarities and the groups were arbitrarily named CHS1 through 7 (Table 2). The mismatch between the arbitrary designation and phylogenetic tree in Fig. 2 arose because the designation was based on nucleotide sequence while the tree was based on the protein sequence. It was not possible to design primers to distinguish each member in a group because of the very high base sequence homology among them. The transcripts of groups 5 through 7 were not detected by qRT-PCR. The transcript levels of all CHS groups, except for CHS4, reached a maximum at 20 days after flowering. The transcript numbers of CHS2 and 4 in the developing seed were considerably low compared to other groups. CHS3 and CHS1 levels, with maxima at 15 and 20 days after flowering respectively, were high enough to correlate with pigment accumulation, although the genes involved in the later-stage of anthocyanin biosynthesis were most active at 10 days after flowering (Fig. 3). Though the primers cannot distinguish individual CHS in a given group, only one gene is known to be expressed each in CHS1 and CHS3, Os05g12210 and Os11g32650, respectively (Table 2). Therefore, a closer examination of the role of those genes in anthocyanin biosynthesis is necessary.

Table 2: Arbitrary grouping of rice putative chalcone synthase in this study. The underlined are known to be expressed

Group No.	Locus Identifier
CHS1	Os01g12180, Os05g12210, Os05g12240, Os10g08620, Os10g08670, Os01g41834
CHS2	Os07g17010, Os07g34140, Os07g34190, Os07g34260, Os11g32620, Os11g32580, Os11g35930
CHS3	Os07g11440, Os11g32610, Os11g32650
CHS4	Os04g01354, Os07g31770
CHS5	Os04g23940, Os10g09860
CHS6	Os07g22850, Os10g07040
CHS7	Os07g31750

The F3'H, F3'5'H, DFR, and ANS transcripts were most abundant on the 10 days after flowering and rapidly diminished to basal level at 20 days after flowering (Fig. 4). However, the transcript level of each gene was very different from gene to gene. While the transcript level of F3'5'H was one-fold lower than F3'H and DFR, whereas ANS level was one-fold higher than F3'H and DFR (Fig. 4). To evaluate if the aforementioned anthocyanidin-related genes follow seed development and maturation, we used GluA-3, a member of the glutelin gene family, as a reference and marker. The pattern of the transcript level of GluA-3 faithfully followed those of F3'H, F3'5'H, DFR, and ANS (Fig. 4).

Anthocyanidin Pigment Content in Black Rice

The anthocyanin content in the developing rice seed was measured to verify the temporal correlation between the

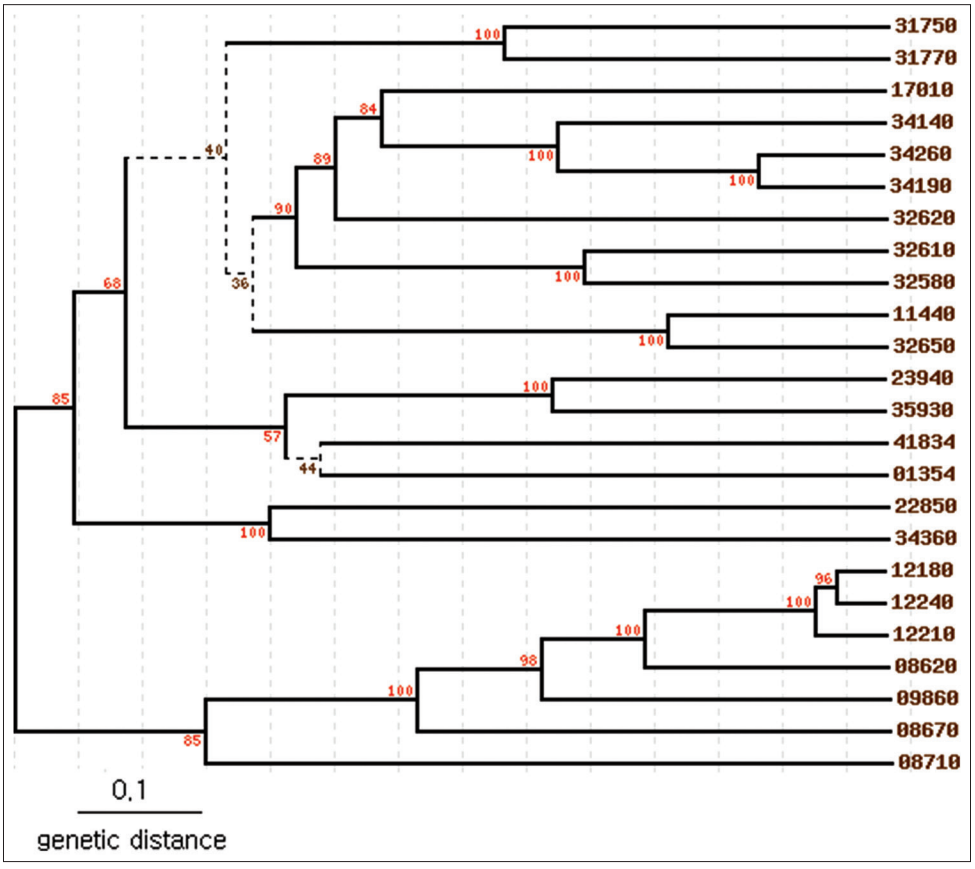

Figure 2: Phylogenetic tree of chalcone synthases in rice

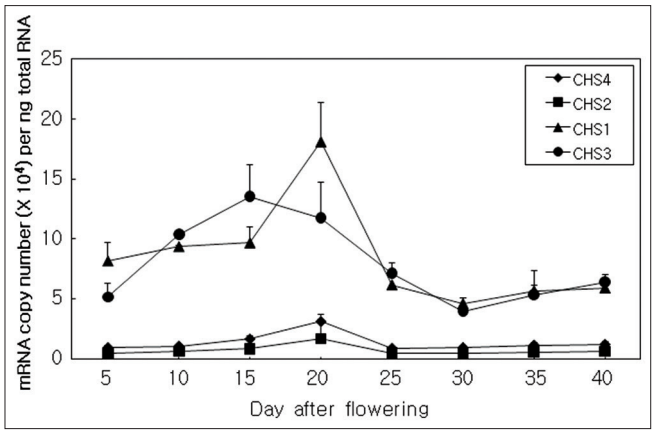

Figure 3: Quantitative real-time PCR transcript levels of chalcone synthase after flowering

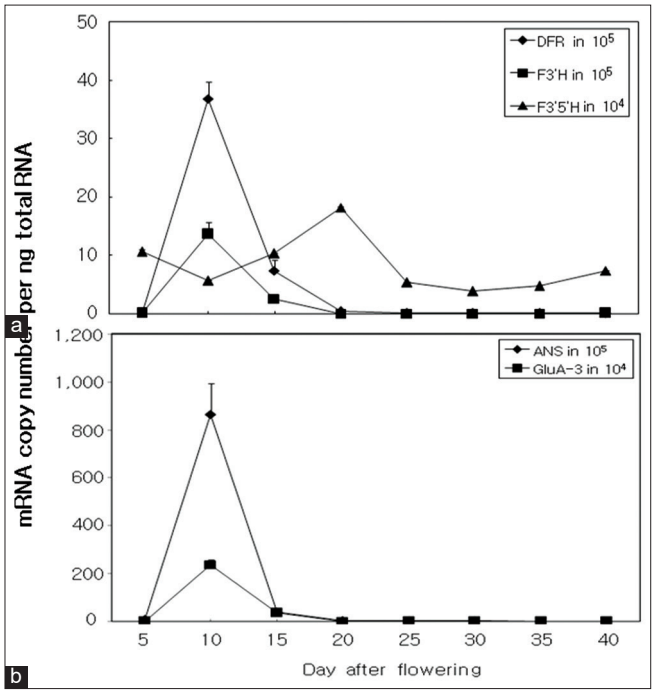

Figure 4: (a and b) Quantitative real-time PCR transcript levels of F3'H, F3'5'H, DFR, ANS, and GluA-3 after flowering

transcript level and the pigment content. Five days after the pigment gene transcript levels reached maxim, that is 15 days after flowering, the pigment content reached the highest value per fresh weight, and then the content gradually decreased (Fig. 5). This timing of increase of anthocyanin content coincided with the pattern of the steady-state transcript level of the anthocyanin-related genes mentioned earlier. The anthocyanin content was reached at a maximum of 92 μg/grain in the next 10 days at a rate of 1.36 μg/grain/day (Fig. 5).

DISCUSSION

Koes *et al.*, 1989 identified twelve copies of CHS genes in petunia. They reported that during flower development CHS-A

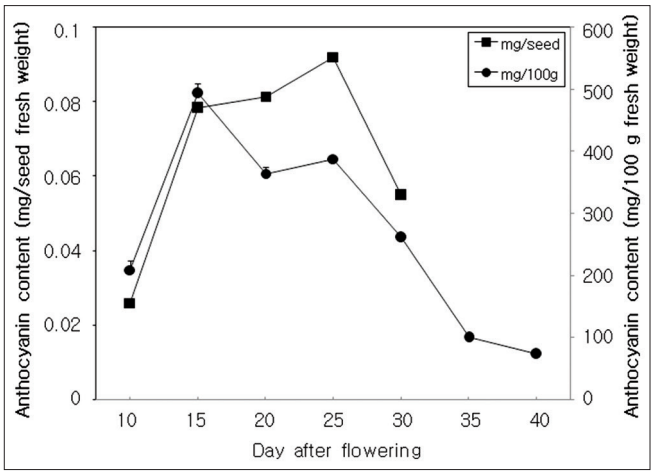

Figure 5: Anthocyanin content analysis with UV-Vis spectrophotometer after flowering

is the key CHS gene expressed, and it might encode for a major CHS protein involved in the anthocyanin biosynthesis. In addition, during flower development, CHS-J is also expressed and the expression level was about 10-fold lower than that of CHS-A (Koes *et al.*, 1989). In black rice, the synthesis of quercetin and cyanidin (Lee *et al.*, 1998), could be separately regulated by isozymes CHS3 and CHS1. The transcript level of CHS2, containing 5 genes known to be transcribed, and CHS4 were too low to be considered as anthocyanin-related.

F3'H and F3'5'H are the two important enzymes in the flavonoid biosynthetic pathway, which leads to the production of colored anthocyanins (cytochrome P450 dependent monooxygenases and anthocyanidin synthase (Forkmann, 1991; Shimada *et al.*, 2005). Takaiwa & Oono, 1991 reported that the GluA-3 gene could be a useful system for studying the temporal-specific expression of seed storage protein genes. The transcript pattern of GluA-3 followed those of F3'H, F3'5'H, DFR, and ANS. This pattern supported the pattern of GluA-3 mRNA level.

The anthocyanin content increased and then the content gradually decreased. If this value was extrapolated with the fresh single grain weight (Yoshida, 1981), the anthocyanin content per grain grew most rapidly from 26 to 78 μg/grain during these 5 days (10 to 15 days after flowering) at a rate of 10.5 μg/grain/day. This timing of increase of anthocyanin content coincided with the pattern of the steady-state transcript level of the anthocyanin-related genes mentioned earlier. The apparent decrease of the pigment level thereafter presumably reflects the rapid conversion of soluble form into polymer-bound insoluble form because the grain weight reached a plateau at 20 days after flowering.

The present results indicated that the gene transcripts for cyanidin biosynthesis in black rice were most abundant at 10 days after flowering and returned to basal level by 20 days after flowering. This temporal change of the pigment-related genes followed the same pattern as the glutelin biosynthetic gene GluA-3 transcript in rice. The behavior of those gene transcript levels was consistent with anthocyanin accumulation

in the seed. One exception to this general pattern was CHS, whose maximal transcript level was reached 5 days later than other genes. It also retained a relatively higher level even after day 20 when other anthocyanidin genes returned to the basal level.

CONCLUSION

To correlate the biosynthesis of the pigments with relevant genes, the expression level of genes putatively involved in the anthocyanin biosynthesis was monitored by qRT-PCR during seed development of black rice. The mRNA level of F3'H, DFR, and ANS, key enzymes in anthocyanidin biosynthesis, peaked at 10 days after flowering. Among the different CHS isoforms, the CHS1 and 3 showed a gradual increase in the transcript level with increasing the day after flowering. The highest level of CHS1 and 3 transcripts result in the highest accumulation of anthocyanidin content at 15 or 20 days after flowering. From these results, it is concluded that the particular CHS isoforms might be responsible for the anthocyanin biosynthesis during seed development. However, in the future, further studies are necessary to determine the complete set of CHS genes in black rice and to analyze how many types of CHS isoforms involved in the anthocyanin biosynthesis during seed development

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AUTHOR'S CONTRIBUTIONS

S.U.K. and S.U.P. designed the experiments and analyzed the data. Y.B.K. and R.S. performed the experiments and analyzed the data. Y.B.K., S.U.K., and S.U.P. wrote the manuscript. All authors read and approved the final manuscript.

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