Physiological factors affecting transcription of genes involved in the flavonoid biosynthetic pathway in different rice varieties

Xiaoqiong Chen1,2, Tomio Itani1, Xianjun Wu2, Yuuki Chikawa1, and Kohei Irifune1,*

1Faculty of Life and Environmental Sciences; Prefectural University of Hiroshima; Shobara, Japan; 2Rice Research Institute; Sichuan Agricultural University; Wenjiang, Sichuan, PR China

Keywords: flavonoid biosynthetic genes, colored rice strains, sucrose, glucose, Oryza sativa L.

Introduction

Rice is generally categorized by caryopsis color into red, green, black, or white varieties. The color of rice is due to the composition of anthocyanin pigments that are ubiquitous throughout the plant kingdom. It is well known that black and red rice possess rather stronger antioxidant capacity because of their rich anthocyanin and proanthocyanin content, which play an important biological role in reducing the risk of oxidative damage, cancer, and cardiovascular disease. These compounds belong to a large class of flavonoids and are derived from a group of phenolic secondary metabolites in plants. All flavonoids possess a basic benzopyrene skeleton, which is synthesized through the sequential condensation reaction of a –coumarone-coenzyme A (-coumaroyl-CoA) and 3 molecules of malonyl-CoA. In addition to subtle modifications in the benzopyrene itself, flavonoids are further diversified by the addition of various moieties to the basic structure, such as hydroxyl and methyl.

A number of studies have described the majority of genes and enzymes involved in the flavonoid biosynthetic pathway of maize, Arabidopsis, and Petunia (Fig. 1). Synthesis of anthocyanin and proanthocyanin utilizes the same early and middle steps of the flavonoid biosynthetic pathway, including chalcone synthase (CHS), chalcone isomerase (CHI), and dihydroflavonol 4-reductase (DFR). Proanthocyanin is synthesized by leucoanthocyanidin reductase (LAR), while anthocyanidin is synthesized by anthocyanidin synthase (ANS). A portion of the anthocyanidin is converted into proanthocyanin by anthocyanidin reductase (ANR) (Fig. 1).

A previous study identified the genes in rice that encode homologs of CHS and CHI based on sequence homology. Similarly, a functional DFR gene was revealed when over expressing transgenic rice was found to produce red-colored pericarp. ANS, a key enzyme in the conversion of colorless leucoanthocyanidin to colored anthocyanidin, was initially isolated from maize, snapdragon, and Petunia. ANS was recently detected in rice by homology research. Chen et al. reported that black rice contains 3 kinds of anthocyanin pigments; however, red rice contains only one micromolecule anthocyanin pigment (malvidin). Moreover, the content of anthocyanin pigment in black rice was 20-fold higher than that in red rice. There was no significant difference in total phenolic content between black and red rice as determined by high-performance liquid chromatography (HPLC). The fact that there are significant differences in the anthocyanin type and content in various rice varieties is interesting. It is possible that variations in anthocyanin type and content have an evolutionary significance for specific plant species. Therefore, the analysis on regulation for some of the putative rice anthocyanin biosynthesis pathway genes could provide novel resources for future research in understanding the flavonoid biosynthetic pathway in rice.
Sugars are a common regulator of gene expression for metabolic enzymes and proteins involved in photosynthesis, carbohydrate metabolism, pathogenesis, and anthocyanin biosynthesis. In addition, Baier et al. reported that the interrelationships between developmental, environmental, and metabolic signal transduction pathways control the production of flavonoids, and anthocyanin biosynthesis was often observed in plants grown on media containing sugar. CHS was induced by sugars in transgenic Arabidopsis leaves, while Petunia corollas cultured in vitro without sucrose (Suc) were devoid of pigment. DFR and ANS were strongly increased by Suc in grape (Vitis vinifera) cells. Additionally, microarray analysis of Arabidopsis pho 3 adult leaves indicated an enhanced expression of the transcription factors Production Of Anthocyanin Pigment 1 (PAP 1) and PAP 2 and Transparent Testa 8 (TT 8), as well as genes encoding anthocyanin biosynthesis enzymes, indicated that sugars are in vivo triggers of anthocyanin biosynthesis. A similar phenomenon has been reported in response to glucose (Glc), which induces flavonoid biosynthesis in Arabidopsis. However, responses to Suc and Glc in rice require further investigation.

Light has been implicated as an important environmental factor regulating plant development and gene expression. Previous studies have shown that anthocyanin pigment accumulation and CHS transcription levels increased in response to light in Arabidopsis seedlings. So far, there has been considerable research regarding the effects of high-intensity white light on gene function and regulation indicating that specific photoreceptors affect gene activation and pigment accumulation induced by visible wavelengths. Moreover, there are researches about induced reaction involved in white light in Arabidopsis, but no reports exist that deal with flavonoid biosynthesis gene expression in colored rice.

The goal of our study was to determine whether white light and sugars affect expression of genes involved in the rice flavonoid biosynthetic pathway. Since flavonoids result in different coloration of the rice grain, such as black, red, green, and white, we also compared the flavonoid content and composition of these distinctly colored rice grains.

Results

Analysis of flavonoid biosynthetic gene transcript levels in different tissues during vegetative growth

Results of analysis of flavonoid biosynthetic gene expression in plants using real-time PCR are shown in Figure 2. The levels of flavonoid biosynthesis gene transcripts varied according to the gene, tissue, and colored rice strain. The expression level of the CHS gene, which catalyzes the first step of the phenylpropanoid pathway leading to flavonoid production (Fig. 1), varied greatly depending on tissue type and colored rice varieties (Fig. 2 CHS). The transcript levels of CHS in the roots of red rice were the highest among the different colored rice varieties. Moreover, significant differences were observed in different tissues of the same colored rice; CHS transcript levels in the roots of red rice was almost 20-fold higher than those of sheath and flag leaf, while CHS expression was barely detected in tillering leaf. Likewise, the roots of green rice presented higher transcript levels than other tissues, such as sheath and flag leaf, but their expression was almost completely absent in tillering leaf. Transcript levels of CHS were lower in all tissues of black rice compared with the other rice strains, and expression was barely detected in flag leaf.

CHI gene expression pattern was found to be similar to that for CHS, with the highest expression observed in the root (Fig. 2 CHI). The CHI transcript levels in roots followed the rank order: green > red > black > white rice, and differed greatly; for example, green rice was 56-fold higher than white rice, and twice that of red rice. However, transcript levels could barely be detected in the tillering leaf of green rice.

DFR was present at significantly higher transcript levels in the roots and sheath of green rice, and the roots of red rice (Fig. 2 DFR). In contrast, its expression was almost absent in tillering leaf of green rice. Significant differences in DFR levels were also observed in the roots of different colored rice strains. Green rice showed a 2-fold greater transcript level than black rice.

Roots of all the rice varieties were found to have the highest expression levels of LAR with highest expression being observed in green rice followed by black, red, and white (Fig. 2 LAR). ANS was present in all tissues of all the rice varieties tested including common (white) rice (Fig. 2 ANS). Besides, its transcript levels were found to be higher in roots than other tissues in red and black rice varieties. These transcript levels were not specific in common rice and were found to be lower in whole plants as expected. Figure 2 showed that roots had the
highest transcript levels of all the anthocyanin genes studied while the tillering leaves showed the lowest expression levels of these genes. In addition, fluctuations in the expression of the 5 flavonoid biosynthesis genes indicated that the transcript levels of genes of flavonoid biosynthesis pathway in root did not coordinate with the down-stream genes (Fig. 3A). This is evident from the transcript level of CHS in the root of red rice, which (5.31) was higher than that of green rice (3.38), whereas, CHI level of red rice was much lower than that of green rice (58.89). We did not observe any significant differences in the expression levels of the gene of down-stream of flavonoid biosynthetic pathway. It can be speculated that the genes are regulated independently of each other, at least for colored rice.

Analysis of transcript levels of flavonoid gene expression during grain filling

Rice 5 flavonoid biosynthesis genes were found to be expressed throughout caryopsis development, and there were not almost varied in different checked stage and tissue in white rice. In contrast, we found significant differences in the transcripts levels of CHS, CHI, DFR, LAR, and ANS in different colored rice varieties tested and at different developmental stages of the same variety (Table 2). For example, the transcript levels of the 5 flavonoid biosynthetic genes of colored rice, in the caryopsis all reached dramatically high levels in the second stage (5 d), with a subsequent decrease in the third stage (7 d). Besides, 5 genes existed in expression difference in different colored rice strains. For example, DFR in the second stages of red rice showed about 7-fold greater transcript levels than that of black rice, and about 160-fold greater transcript levels than that of the other colored rice strains. ANS levels in the second development stage of black rice were more than 8-fold and 5-fold higher than red and green rice, respectively. LAR transcript levels in red rice were the highest among the colored rice. Gene transcript levels in the different development stages were found
to significantly differ from 44-fold to hundreds-fold in black rice, while CHI in the second stage was about 2 – and 19-fold greater than the first and third stages, respectively. Moreover, this data shows that the expression levels of ANS and LAR, which are derived from the common up-stream gene DFR in the flavonoid biosynthesis pathway, are correlated; for example, Table 2 shows that during conditions resulting in elevated expression of DFR, LAR (767.12) transcription was 14-fold higher than that of ANS (51.65) in red rice. In contrast, the ANS (419.23) transcript level was about 4-fold higher than that of LAR (102.12) in black rice. Fluctuations in the expression of the 5 flavonoid biosynthesis genes are regulated independently of each other as expression of root (Fig. 3B). Therefore, it can be further speculated that the genes are regulated independently of each other in rice.

Flavonoid biosynthesis gene expression levels varied in the endosperm of different colored rice strains (Table 2). The levels of expression in green and black rice were higher than those in white rice, while there was hardly expression in red rice.

Expression of flavonoid biosynthesis genes in response to sugars

This study analyzed 5 flavonoid biosynthesis genes used black rice response to sugar. The results showed that the expression of DFR, LAR, and ANS are strongly induced in response to Suc (Fig. 4). For example, 0.2 M Suc strongly induced DFR and ANS transcription levels to about 7 – and 4-fold greater than control, respectively. Besides, flavonoid biosynthesis genes presented in a dose dependent manner for different genes. For example, treatment with 0.4 M Suc resulted in the highest LAR, CHI, and CHS transcript levels, while treatment with 0.2 M Suc presented the highest level for DFR and ANS. Glc was found to strongly repress transcription of all the genes in this study. For example, DFR showed 50-fold lower expression levels with Glc and a 200-fold lower expression in response to treatment with a mixture of Glc and Suc. Therefore, Glc was found to negatively regulate flavonoid biosynthesis gene transcription.

Expression of flavonoid biosynthesis genes in response to white light

White light was found to induce the transcription of all of the flavonoid biosynthetic genes, with exposure-dependent differences observed (Fig. 5). DFR, LAR, ANS, and CHI transcript levels with 24-h exposure were greater than those at 16 h. In contrast, CHS transcript levels at 16 h were greater than those observed at 24 h. Moreover, differences were observed in the transcript levels of genes with exposure to white light; for example, white light strongly affected DFR expression, increasing 8 – and 9-fold at 16 and 24 h, respectively. Likewise, maximal CHS expression was observed at 16 h (7-fold greater than control). Subsequently, levels decreased with increasing exposure time; CHS at 24 h was 5-fold higher than that of control.

Discussion

Low transcription levels for the 5 flavonoid biosynthesis genes were observed in all white rice tissues as expected, and these levels showed minimal variation, especially during caryopsis formation (Fig. 2). Therefore, the low flavonoid content and colorlessness of white rice is due to the lower expression of flavonoid biosynthetic pathway genes. In contrast, significant differences in gene expression were observed among the 3 colored rice varieties, and occurred to specificity for spatial and temporal. The DFR gene was detected in overexpressing transgenic rice observed to produce red-colored pericarp. This concurs with the results from the study by Furukawa et al.,13 that the red pericarp is derived mainly from the strong expression of DFR. Red rice was also found to

---

Table 1. List of RT-PCR primers for flavonoid genes

| Locus ID   | Primer    | Sequence (5’→ 3’)                                      | Product size/bp |
|------------|-----------|--------------------------------------------------------|-----------------|
| CHS AC136226 | F R      | CATGGAATTTTGGGGATTGG AATTGTTCGA GCAGACACTG             | 226             |
| CHI XM-470129 | F R      | AGAAGTTCA CAGGGTTGACG GAGTGGGTA GAGAGATGA            | 195             |
| DFR Y07956  | F R      | GTCAAGTAT GCGGATGGAT AACTGCACT GCATCGAGAT            | 217             |
| ANS Y07955  | F R      | CTCCTTCTG GGTCGTTTCTTGC TATCATCGGT TCTG             | 184             |
| LAR BN000704 | F R      | CTTGATGTGCA ACTCCA GTGAGCGATC TTGTTGATGC          | 195             |

---

Figure 3. Transcription levels of flavonoid biosynthesis-related genes in different colored rice strains. Note: (A) Transcription levels of flavonoid biosynthesis-related genes in root of different colored rice strains. (B) Transcription levels of flavonoid biosynthesis-related genes in the second stage of caryopsis development of different colored rice strains.
contain a rather high LAR (767.12) transcription level while black rice showed a relatively high ANS (419.23) transcription level compared with LAR. The difference in expression levels of ANS in black and red rice corroborates the results of Chen et al.35 that black rice contain rich anthocyanin pigments derive from higher ANS expressions. This study may speculate red rice contains higher proanthocyanin content because of higher expression of LAR. Low transcription levels of the 5 flavonoid biosynthesis genes were detected in white rice, and high levels were found in red and black rice. Therefore, the color of rice grains is governed by their phenolic content, which is higher in black and red rice as compared with white and green rice. Furthermore, our study shows that the endosperm of all colored rice varieties has lower transcript levels of the flavonoid biosynthetic genes as compared with the seed capsule resulting in lower levels of antioxidants in the endosperm. This information is vital to studies that aim to extract anthocyanins from plants for medicinal purposes. Higher transcript levels of the 5 flavonoid biosynthetic pathway genes among colored rice varieties indicate the possibility that a strong promoter or other factors that induce the up-stream gene transcription of the flavonoid biosynthetic pathway exist in these rice varieties. The excessive accumulation of anthocyanin and proanthocyanin in seed capsule of black and red rice is likely driven by this promoter. It has been previously reported that *long hypocotyl 5* (a Leu-zipper TF) and phytochrome-interacting transcription factor 3 (PIF3) binds directly to the promoters for anthocyanin structural genes, such as CHS, CHI, F3H, DFR, and LDOX, in *Arabidopsis*.33,34 Further research is required to elucidate the triggers responsible for the dramatic increase in flavonoid biosynthetic gene transcription in rice. Reddy et al.14 reported that increased accumulation of anthocyanin was accompanied by a concomitant decrease in proanthocyanidin. Consistent with these results, our results demonstrate a strongly competitive mechanism between anthocyanin and proanthocyanidin (Fig. 6). These results further explain the high anthocyanin content of seed capsule of black rice, and the rich proanthocyanin content of seed capsule of red rice. The expression of anthocyanin biosynthetic genes appears to be highly coordinated in grape skin during berry development and in bilberry fruit development.35 Similar to the result for grape and bilberry, direct associations between CHS, CHI, and DFR in the dicot model plant *Arabidopsis* and the flux of intermediates into different flavonoid products were found to be controlled by the formation of metabolons.36,37 However, Clive-Lo and Nicholson25 reported that the expression of flavonoid biosynthesis genes was not coordinated in *sorghum* mesocotyl. Our study reiterates the findings of Clive-Lo and Nicholson25 by demonstrating that the 5 flavonoid biosynthesis genes analyzed were independently expressed in monocot rice (Fig. 3). Presumably significant differences exist between monocot and dicot expression modules, at least with respect to rice.

Several studies have investigated the sugar-induced accumulation of anthocyanins. The genes encoding DFR and ANS were found to be upregulated, and anthocyanin accumulation was significantly increased by exposure to Suc in grape cells.36 Solfanielli et al.36 reported that flavonoid and

| Gene expression (2-ΔΔct) of caryopsis* | Gene expression (2-ΔΔct) of endosperm* |
|--------------------------------------|----------------------------------------|
| CHS                                  |                                        |
| White riceb                          | 1                                      |
| Green rice                           | 1                                      |
| Red rice                             | 1                                      |
| Black rice                           | 1                                      |
| CHI                                   |                                        |
| White riceb                          | 1                                      |
| Green rice                           | 1                                      |
| Red rice                             | 1                                      |
| Black rice                           | 1                                      |
| DFR                                   |                                        |
| White riceb                          | 1                                      |
| Green rice                           | 1                                      |
| Red rice                             | 1                                      |
| Black rice                           | 1                                      |
| ANS                                   |                                        |
| White riceb                          | 1                                      |
| Green rice                           | 1                                      |
| Red rice                             | 1                                      |
| Black rice                           | 1                                      |
| LAR                                   |                                        |
| White riceb                          | 1                                      |
| Green rice                           | 1                                      |
| Red rice                             | 1                                      |
| Black rice                           | 1                                      |

Note: * Values expressed as means ± SD (n = 3). White rice = expressed as control. nd described rather lower expression level. RNA of caryopsis were extracted from 3, 5, and 7 d after flowering. RNA of endosperm were extracted from 5 d after flowering. Transcript levels were quantified by reverse transcription (RT)-qPCR (for details, see Materials and Methods).

---

*www.landesbioscience.com Plant Signaling & Behavior e27555-5*
anthocyanin biosynthesis pathway genes were strongly upregulated following Suc treatment, affecting both flavonoid and anthocyanin contents in Arabidopsis. This was particularly evident for CHS, CHI, and DFR in this study. Excised Arabidopsis leaves showed anthocyanin accumulation and increased levels of CHS, CHI, and DFR mRNAs in response to Suc supplementation. The expression of grape DFR was found to be responsive to Suc, but not Glc and Fru, was found to induce PAP1 and ANS mRNA levels in Arabidopsis and trigger a dominant increase in anthocyanin synthesis. In contrast, Gollop et al. reported that the induction of anthocyanin biosynthetic genes by sugars was rather unspecific, and Glc and Fru as well as Suc treatments resulted in the induction of DFR and ANS in grape. Similar to Solfanelli’s result in rice, our study showed that Suc significantly induced the transcription of CHS, CHI, DFR, ANS, and LAR. Glc 0.2 M and 0.6 M and a mixture of Suc and Glc were found to negatively regulate flavonoid biosynthetic genes (Fig. 4). It is reasonable to assume that the sugar-specific mechanisms active exist difference in different plants, such as non-specific sugar responses in grape, and all induce anthocyanin biosynthesis and increase DFR expression. However, specific sugar responses exist in Arabidopsis and rice, and only Suc was able to strongly induce the transcription of flavonoid biosynthetic genes. Besides, the lower levels of gene transcripts in endosperm may be attributed to the suppression of flavonoid biosynthetic genes transcription by Glc in the endosperm.

Solfanelli et al. reported that downstream genes in the flavonoid biosynthetic pathway were induced several hundred-fold by Suc, whereas genes upstream of DFR showed a lower induction. In this study, the effect of Suc on flavonoid biosynthetic pathway gene expression did not appear to be region dependent, as expression levels were largely the same for the whole biosynthetic pathway. Therefore, it is possible that differences in the regulatory elements of up-stream genes involved in sugar responses exist in different plants. Jeong et al. reported that ethylene inhibits anthocyanin accumulation in Arabidopsis seedlings grown in the presence of Suc. The Arabidopsis phe 3 mutant, which has a defective copy of the Suc transporter (Suc 2) gene (encoding a phloem-loading Suc-proton symporter) leading to the accumulation of soluble sugars and starch, showed growth retardation and anthocyanin accumulation. Expression of the flavonoid biosynthesis pathway transcription factors PAPI and PAP 2 result in enhanced CHS and DFR expression in Arabidopsis. In Arabidopsis, DFR expression is under the control of TT2, which interacts with TT8. Understanding the mechanism due to which up-stream factors stimulate or suppress the accumulation of anthocyanins in response to Suc and Glc in rice continues to intrigue geneticists.

In UV-B responses, a very short period of exposure could induce anthocyanin biosynthesis in rice. Our research showed that white light could induce transcription of anthocyanin biosynthetic gene, but it is not stronger. Therefore different light sources do affect the transcription levels of the gene ANS in rice. Moscoviet al. reported that light was essential for ANS and CHS expression in the detached corolla of petunia flowers. CHS gene induction showed a time-dependent increase during 24 h UV-A exposure in swollen hypocotyls of the red turnip “Tsuda” and anthocyanin accumulation was induced. F3H, DFR, and ANS gene expression in sorghum mesocotyl dramatically decreased with light treatment, whereas that for CHS significantly increased. It was observed that in Arabidopsis cell culture under low fluence white light resulted in very low CHS transcript levels induced in mature leaf tissue. The flavonoid biosynthetic genes

Figure 4. Effect of sugar treatment on flavonoid biosynthesis-related gene transcription levels. Note: Error bars represent SD values for the means of 3 biological replicates, each determined in triplicate.

Figure 5. The effect of white light treatment (12, 16, and 24 h) on biosynthesis-related gene transcript levels. Note: Error bars represent SD values for the means of 3 biological replicates, each determined in triplicate. Twelve h expressed as control.

Figure 6. Comparison of expression levels of downstream genes in flavonoid biosynthesis pathway between red and black rice.
CHS and CHI showed high transcript abundance in high-light Mitchell plants, with weak signals observed for DFR and ANS. In contrast, strong signals for DFR and ANS were detected in Lc plants from Petunia axillaris × (Petunia axillaris × Petunia) hybrid cv “Rose of Heaven.” Our study showed that white light could induce the transcription of the entire flavonoid biosynthetic gene pathway; however, differences were observed in the degree of sensitivity and the required illumination time. Moreover, the flavonoid biosynthesis pathway response in different plants varies with the illumination source, at least for rice. Our results concur with the previous studies in Arabidopsis and demonstrate that light differentially regulates the genes of the flavonoid biosynthetic pathway. The differences in regulation of the flavonoid biosynthetic pathway are light dependent. Previous studies have shown that higher light intensities significantly stimulate CHS and CHI transcript levels while DFR and ANS were not sufficiently stimulated. Our study demonstrated that white light stimulates the DFR transcripts more than any of the other flavonoid biosynthetic genes, such as LAR, ANS, CHI, and so on. Our research paves the way for further understanding of the flavonoid biosynthetic pathway and provides novel resources for research on secondary metabolism in rice, especially on the elucidation of the processes involved in modulating antioxidant content in rice at a molecular level.

Material and Methods

The following japonica rice (Oryza sativa L.) lines were used for expression analysis of flavonoid biosynthesis genes in distinctly colored rice strains: white rice Nipponbare (improved, Japan), black rice Chintakurumai (semi-improved, China), red rice Tsukushiaakamochi (improved, Japan), green rice Akunemochi (native, Japan), and were grown under natural conditions in the experimental fields of the Faculty of Life and Environmental Sciences, Prefectural University of Hiroshima, from May to September 2011. Root RNA was extracted from a mixture of late tillering stage tip and middle sections of the root. Sheath RNA was extracted during the early tillering stage. Tillerling leaf RNA was extracted at the same time as the sheath RNA. Flag leaf RNA was extracted 7 d after flowering. Caryopsis RNA was extracted at 3, 5, and 7 d after flowering, and endospermRNA was extracted at 7 d after flowering. Black rice (Chintakurumai) was grown in potting soil in a green house for 30 d; a portion of the plants were used for light treatment.

Sugar treatments

Rice plants were treated with various concentrations of Suc (0, 0.2, 0.4, 0.6 M), Glc (0.2, 0.6 M), or a mixture of Suc 0.2 M and Glc 0.2 M for 30 h, and each treatment was repeated three times. RNA was extracted from samples treated with sugar solutions for 30 h.

Light treatments

The plants were transferred to a controlled environment growth cabinet, arranged randomly within the cabinet and spaced to prevent shading. Plants were grown in 12, 16, or 24 h photoperiods (6660 lx), at a constant 25 °C and 65% humidity for a period of 3 d. The white light in the growth cabinet was provided by NK Nippon Medic AL 8 Chemical Instruments Co., Ltd (Tokyo, Japan). RNA was extracted at the end of the treatment period.

Real-time reverse transcription-qPCR analysis

Total RNA was extracted using a Trizol RNA mini-kit following the manufacturer’s protocol (Qiagen, Valencia, CA, USA). cDNA was synthesized from 1 μg of total RNA using the iScript cDNA Synthesis Kit (Bio-Rad). qPCR was performed using the MX3000P Real Time System (Agilent Technologies, Inc, CA, USA), following the manufacturer’s instructions. All reactions were performed with a Brilliant III Ultra-Fast SYBR Green QPCR Kit (Finzymes, Agilent Technologies, CA, USA), according to the manufacturer’s instructions. PCR reactions were performed in triplicates with 10 μl of 2 × SYBR Green QPCR master mix, 1 μM of each primer (Table 1), 1 μl of 10-fold-diluted cDNA, 0.3 μl of diluted reference dye (30 nM), and nuclease-free water to a final volume of 20 μl. A negative control (water) was included in each run. Reactions were incubated at 95 °C for 3 min, followed by 40 cycles of amplification at 95 °C for 20 s and then 60 °C for 20 s, after which a final extension step was performed at 72 °C for 1 min. Fluorescence was measured at the end of each extension step. Amplification was followed by melting curve analysis with continual fluorescence data acquisition during the 65 °C to 95 °C melt. The raw data were analyzed with CFX Manager software and expression was normalized to that of the rice actin gene (forward primer: TGAGACACCTACCGGAATC; reverse primer: ATCCCCGACCAATCTGGA) to minimize variation in cDNA template levels. Relative expression levels were calculated using the comparative threshold (Ct, cycle value) method. Mean values were obtained from 2 biological replicates, each determined in triplicate.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

References

1. Oki T, Masuda M, Kohayashi M, Nishiba Y, Furuta S, Suda I, Sato T. Polymeric procyanidins as radical-scavenging components in red-hulled rice. J Agric Food Chem 2002; 50:7524-9; PMID:12475265; http://dx.doi.org/10.1021/jf025841z
2. Russo A, La Fausi L, Acquaviva R, Campisi A, Racin G, Scifo C, Renis M, Galvano G, Vanella A, Galvano F. Ochratoxin A-induced DNA damage in human fibroblast: protective effect of cyanidin 3-O-beta-d-glucoside. J Nutr Biochem 2005; 16:31-7; PMID:15629238; http://dx.doi.org/10.1016/j.jnutbio.2004.05.005
3. Barron D, Ibrahim RK. Isoprenylated flavonoids: a survey. Phytochemistry 1996; 43:921-82; http://dx.doi.org/10.1016/S0031-9422(96)00344-5
4. Tahara S, Ibrahim RK. Prenylated isoflavonoids: an update. Phytochem 1995; 38:1073-94; http://dx.doi.org/10.1016/0031-9422(94)00788-U
5. Holton TA, Cornish EC. Genetics and biochemistry of anthocyanin biosynthesis. Plant Cell 1995; 7:1071-83; PMID:12242498
6. Mol J, Grotenhuis E, Koos R. How genes paint flowers and seeds. Trends Plant Sci 1998; 3:212-7; http://dx.doi.org/10.1016/S1369-5266(98)01242-4
7. Springborg K, Nakajima J, Yamazaki M, Saito K. Recent advances in the biosynthesis and accumulation of anthocyanins. Nat Prod Rep 2003; 20:288-303; PMID:12828368; http://dx.doi.org/10.1039/b109542k
8. Weishaar B, Jenkins GI. Phenylpropanoid biosynthesis and its regulation. Curr Opin Plant Biol 1998; 1:251-7; PMID:10066590; http://dx.doi.org/10.1016/S1369-5266(98)80113-1
9. Maides MAS, Ray H, Gruber MY. New perspectives on proanthocyanidin biosynthesis and molecular regulation. Phytochemistry 2003; 64:367-83; PMID:12943753; http://dx.doi.org/10.1016/S0031-9422(03)00377-7
10. Winkel-Shirley B. Flavonoid biosynthesis. A colorful model for genetics, biochemistry, cell biology, and biotechnology. Plant Physiol 2001; 126:485-93; PMID:11402719; http://dx.doi.org/10.1104/pp.126.2.485

11. Orska A, Kudrna D, Rostoks N, Brueggeman V, von Wettstein D, Kleinhofs A. Chlorone isomerase gene from (Oryza sativa) and barley (Hordeum vulgare): physical, genetic and mutation mapping. Gene 2003; 302:171-8; PMID:12527208; http://dx.doi.org/10.1016/s0378-1119(02)01015-3

12. Reddy AR, Scheffler B, Madhuri G, Srivastava MN, Kumar A, Sarbanyaravanan PV, Nair S, Mohan M. Chlorone synthase in rice (Oryza sativa L.): detection of the CHS protein in seedlings and molecular mapping of the chs locus. Plant Mol Biol 1996; 32:735-43; PMID:8980525; http://dx.doi.org/10.1007/BF00020214

13. Furukawa T, Maekawa M, Oka T, Suda I, Iida S, Shima H, Takamura I, Kadowaki K. The Rc and Rd genes are involved in proanthocyanin synthesis in rice pericarp. Plant J 2007; 49:91-102; PMID:17163879; http://dx.doi.org/10.1111/j.1365-313X.2006.02958.x

14. Reddy AM, Reddy VS, Scheffler BE, Wientan U, Reddy AR. Novel transgenic rice overexpressing anthocyanidin synthase accumulates a mixture of flavonoids leading to an increased antioxidative potential. Metab Eng 2007; 9:95-111; PMID:17157544; http://dx.doi.org/10.1016/j.ymben.2006.09.003

15. Chen XQ, Nagaog N, Irani T, Irfune K. Anti-oxidative activity, and identification and quantification of anthocyanin pigments in different coloured rice. Food Chem 2012; 135:2783-8; PMID:22928872; http://dx.doi.org/10.1016/j.foodchem.2012.06.098

16. Rolland F, Baena-Gonzalez E, Sheen J. Sugar sens- ing and signaling in plants: conserved and novel mechanisms. Annu Rev Plant Biol 2006; 57:675-709; PMID:16669778; http://dx.doi.org/10.1146/annurev.arplant.57.032905.105441

17. Baier M, Hemmann G, Holman R, Corke F, Card R, Smith C, Roos F, Bevan MW. Characterization of mutants in Arabidopsis showing increased sugar-specific gene expression, growth, and develop- mental responses. Plant Physiol 2004; 134:81-91; PMID:14684841; http://dx.doi.org/10.1104/pp.103.031674

18. Tsuikaya H, Ohshima T, Naito S, Chino M, Komeda H. Expression of the grape dihydroflavonol reductase gene for chalcone synthase from petunia in transgenic Arabidopsis cells. Plant Cell 1996; 8:1555-67; PMID:8837509

19. Batschauer A. Roach Mo, Kaiser T, Nagatani A, Fursa M, Schafer E. Blue and UV-A light-regulated expression of chimeric chalcone synthase gene in Arabidopsis cells. Plant Cell 1996; 8:1555-67; PMID:8837509

20. Feinbaum RL, Storz G, Ausubel FM. High inten- sity and blue light regulated expression of chimeric chalcone synthase genes in transgenic Arabidopsis thaliana plants. Mol Gen Genet 1991; 226:449-56; PMID:2038307; http://dx.doi.org/10.1007/BF00260658

21. Kubacek W, Shirley BW, McKillop A, Goodman HM, Briggs W, Ausubel FM. Regulation of flavonoid biosynthetic genes in germinating Arabidopsis seedlings. Plant Cell 1992; 4:1229-36; PMID:12297632

22. Pelletier MK, Shirley BW. Analysis of flavo- noid 3-hydroxylase in Arabidopsis seedlings. Coordination regulation with chalcone synthase and chlorone isomerase. Plant Physiol 1996; 111:339-45; PMID:8685272; http://dx.doi.org/10.1104/pp.111.1.339

23. Lee J, He K, Stole V, Lee H, Figueroa P, Lee H, Tongtatsin W, Zhao H, Lee I, Dong XW. Analysis of transcription factor HYS genomic binding sites revealed its hierarchical role in light regulation of development. Plant Cell 2007; 19:731-49; PMID:17337630; http://dx.doi.org/10.1016/j.ppc.2006.04.088

24. Shin J, Park E, Choi G. PIF3 regulates anthocy- anin biosynthesis in an HYS-dependent manner with both factors directly binding anthocyanin biosynthetic gene promoters in Arabidopsis. Plant J 2007; 49:981-94; PMID:17339887; http://dx.doi.org/10.1111/j.1365-313X.2006.03021.x

25. Pertel MK, Berulis IE, Winkel-Shirley B. Disruption of specific flavonoid genes enhances the accumulation of flavonoid enzymes and end-products in Arabidopsis seedlings. Plant Mol Biol 1999; 40:45-54; PMID:10394944; http://dx.doi.org/10.1023/A:1026414010100

26. Nita S, Murano N, Akaike M, Nakamura K. Mutants of Arabidopsis thaliana with pleiotropic effects on the expression of the gene for β-amylase and on the accumu- lation of anthocyanin that are inducible by sug- ars. Plant J 1997; 11:841-51; PMID:9161039; http://dx.doi.org/10.1046/j.1365-313X.1997.11040841.x

27. Zhou B, Li X, Xu Z, Yan H, Homma S, Kawaiwa S. Ultraviolet A-specific induction of anthocyanin bio- synthesis in the swollen hypocotyls of turnip (Brassica rapa) J Exp Bot 2007; 58:1771-81; PMID:17426056; http://dx.doi.org/10.1093/jxb/erm036

28. Feinbaum RL, Ausubel FM. Transcriptional regu- lation of the Arabidopsis thaliana chalcone synthase gene. Mol Cell Biol 1988; 8:1985-92; PMID:3386631

29. Jackson JA, Fuglevand G, Brown BA, Shaw MJ, Jenkins GI. Isolation of Arabidopsis mutants altered in the light-regulation of chalcone synthase gene expression using a transgenic screening approach. Plant J 1995; 8:369-80; PMID:7503755; http://dx.doi.org/10.1046/j.1365-313X.1995.0003069.x

30. Albert NW, Lewis DH, Zhang H, Irving JJ, Jameson PE, Davies KM. Light-induced vegetative anthoc- yanin pigmentation in Petunia. J Exp Bot 2009; 60:2191-202; PMID:19580425; http://dx.doi.org/10.1093/jxb/erp097

31. Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. Methods 2001; 25:402-8; PMID:11866690; http://dx.doi.org/10.1016/s1046-8023(01)026-a