Identification and Antioxidant Capacity of Anthocyanin Pigment, and Expressional Analysis of Flavonoid Biosynthetic Genes in Colored Rice Strains

Academic Dissertation

Xiao Qiong Chen

March 2013

Contents

Table of contents……1

List of figures…….....8

List of tables……10

Abstract.....12

Chapter 1: Introduction……16

1. Flavonoid……....16

1.1. Structure of flavonoid……..16

1.2. Biosynthetic pathway of flavonoid……..17

2. Progress of anthocyanin researched……...18

2.1. Structure and classification of anthocyanin……18

2.2. Physiological function of anthocyanin……...20

2.3. Factors of anthocyanin affected……19

2.4. Expression of anthocyanin pigments in rice……...19

3. Aim of research…….…….21

Chapter 2: Identification and qualification of anthocyanin pigment in different colored rice strains……..…23

1. Introduction……..……..23

2. Materials and methods……..……..25

2.1. Materials……23

2.2. Chemicals……..24

2.3. Sample preparation……24

2.4. Measurement of total anthocyanin content andidentification of anthocyanin……..……24
2.5. Oxygen radical absorbing capacity (ORAC) assay……...24
2.6. Statistical analysis…….….25
3. Results and discussion……25
4. Conclusions……29
Chapter 3: Measurement of polyphonic contents in different colored rice strains……..30
1. Introduction……30
2. Materials and method…….30
2.1. Materials…….30
2.2. Chemicals……...30
2.3. Sample extraction……...31
2.4. Measurement of total phenolic content (TPC)……..….31
2.5. Statistical analysis……..31
3. Results and discussion……32
4. Conclusion……..33
Chapter 4: Antioxidant analysis in different colored rice strains……..34
1. Introduction……34
2. Materials and methods……35
2.1. Materials.....35
2.2. Chemicals.....35

2.3. Sample preparation……36

2.4. Oxygen radical absorbing capacity (ORAC) assay……...36

2.5. Statistical analysis……..37

3. Results and discussion……..37

3.1. Antioxidant capacity of the caryopsis……..37

3.2. Antioxidant capacity of the endosperm……...39

3.3. Antioxidant activity of authentic anthocyanins……...40

3.4. Antioxidant capacity of anthocyanin pigments of colored rice strains……...40

4. Conclusion…….…….…..42

Chapter 5: Comparison of antioxidant capacities and phenolic content among colored rice strains using various solvents and extraction method…….….44

1. Introduction……..44

2. Materials and methods…….46

2.1. Materials……..46

2.2. Chemicals……46

2.3. Sample preparation……..……47

2.4. Statistical method of meteorological environment factors…….47

2.5. DPPH method……..48

2.6. ORAC method…….49

2.7. Measurement of total phenolic content (TPC)……..…..49

2.8. Measurement of color……..……50

3. Results and discussion……..……..……..50

3.1. Color of different colored rice strains……..……50

3.2. Comparison of antioxidant capacity in different colored rice strains using different extraction methods…….....52

3.3. Antioxidant capacity determined by the ORAC assay using different colored rice strain……...…54

3.4. Antioxidant capacity in different planting years in different colored rice strains determined by DPPH assay……...57

3.5. Comparison of total phenolics content (TPC) with different extraction methods in different colored rice strain……..…….……58

3.6. Comparison of total phenolics content (TPC) for different year of planting in different colored rice strains…….60

3.7. Correlation between TPC and factors of meteorological environment……..……..……..…….61

3.8. Correlation between TPC and antioxidant capacity……...62

4. Conclusion……..…….…….……62

Chapter 6: Expressional analysis of flavonoid biosynthetic genes

indifferent colored rice strains…….……64

1. Introduction……..65

2. Materials and methods……..65

2.1. Plant material and growth conditions……65

2.2. RNA extraction……..66

2.3. cDNA transcription……67

2.4. Real-time Reverse Transcription-qPCR Analysis…….68

3. Results and discussion……...69

3.1. Analysis of flavonoid biosynthetic gene transcript levels in different tissues during vegetative growth……..….69

3.2. Analysis of transcript levels of flavonoid gene expression during grain filling……73

3.3. Discussion……... 76

4. Discussion……..78

Chapter 7: Sugar inducing flavonoid biosynthetic genes response……..…79

1. Introduction……..80

2. Materials and methods…….……80

2.1. Plant material and growth conditions……....80

2.2. Sugar treatment……..80

2.3. RNA extraction……..80

2.4. cDNA transcription……80

2.5. Real-time Reverse Transcription-qPCR Analysis….80

3. Results and discussion……..81

3.1. Result…….81

3.2. Discussion……..82

4. Conclusion……84

Chapter 8: White light inducing flavonoid biosynthetic genes response……..85

1. Introduction……85

2. Materials and methods……85

2.1. Plant material and growth conditions……85

2.2. Light treatment……..86

2.3. RNA extraction…….…….86

2.4. cDNA transcription……86

2.5. Real-time Reverse Transcription-qPCR Analysis….86

3. Results and discussion……86

3.1. Result…….86

3.2. Discussion…….…….88

4. Conclusion…… 89

Conclusion……90

References……91

Acknowledgement……. 104

List of figures

Fig.1.1. Basic structure of flavonoid compounds R_1, R_2, R_3, and R_4 representing radicles that can be modified.....16

Fig.1.2. Synthetic process of flavonoid.....17

Fig.1.3. Structure of caryopsis.....20

Fig.1.4. Four kinds of colored rice strains.....21

Fig.2.1. Typical chromatogram of standard anthocyanin pigments and anthocyanins of two black and one red rice cultivars.....27

Fig.3.1. Total phenolic content of different colored rice cultivars.....32

Fig.4.1. Evaluation of the antioxidant capacities of four standard anthocyanins by ORAC assay……40

Fig.5.1. Evaluation of the antioxidant capacities of three different colored rice strains planted in 2007 by ORAC assay……..56

Fig.5.2. Correlation of four kinds of colored rice strains planted 2007 between DPPH and ORAC…….....……56

Fig.6.1. Transcript levels of flavonoid biosynthesis-related genes in different tissues of four colored rice strains.....71

Fig.6.2. Transcript levels of flavonoid biosynthesis-related genes in the roots of four colored rice strains.....73

Fig.7.1. The effect of sugar treatment on flavonoid biosynthesis-related gene transcript levels.....81

Fig.8.1. The effect of white light treatment (12, 16 and 24 h) on

biosynthesis-related gene transcript levels.....87

List of tables

Table 2.1. Anthocyanin content and ratios in four different colored rice groups.....28

Table 4.1. Oxygen radical absorbing capacity (ORAC) of rice caryopsis and endosperm[A].....38

Table 4.2. Oxygen radical absorbing capacity (ORAC) of four kinds of anthocyanin pigments and caryopsis anthocyanin contribution to ORAC.....42

Table 5.1. Climatic index from after flowering to harvest…….....48

Table 5.2. Hunters reflected color values……..…..….51

Table 5.3. Antioxidant capacity determined by DPPH assay after extraction with different solvents in five rice strains planted in 2007…….....54

Table 5.4. Antioxidant capacity determined by DPPH method after extraction with 1% HCl methanol in five rice cultivars……57

Table 5.5. Relationship between meteorological condition and color hue or antioxidant capacity or total phenolic content (TPC) in Tsukushiakamochi…….…….58

Table 5.6. Phenolics content measured by UV spectrophotometry after extraction with different solvents in five rice strains planted in 2007.....60

Table 5.7. Phenolics content obtained by extraction with 1% HCl methanol in five rice strains in different years of planting.....61
Table 6.1. List of RT-PCR primers of flavonoid genes.....69
Table 6.2. Expression of flavonoid biosynthetic genes in caryopsis and endosperm.....75

Abstract

Rice (*Oryza sativa* L.) is generally categorized into red, black, and white (common) varieties by caryopsis color at harvest time, which is determined by the composition of flavonoids such as anthocyanin. Black rice is rich in anthocyanin and red rice contains relatively larger amount of proanthocyanidin. These compounds belong to a large class of flavonoids and are derived from a group of phenolic secondary metabolites in plants. Therefore, colored rice has many physiological function, and has attracted considerable research in the past decades, such as, antioxidant capacity, anticancer and so on.

Chapter 1 is an introduction describing the purpose of the thesis. There has been considerable research about content and type of anthocyanin pigments in colored rice, but results differ with the study. Also, there is little information on gene regulation of flavonoid biosynthetic pathway of colored rice. Therefore, the objectives of this study are as follows: 1) Identification and quantification of anthocyanin pigments of the colored rice, 2) antioxidant capacity of caryopsis and endosperm, and contribution of anthocyanin pigments to antioxidant capacity, and 3) expression analysis of genes concerning biosynthesis of anthocyanin pigments.

In chapter 2, anthocyanin pigments of black and red rice were isolated and identified using high-performance liquid chromatography techniques. The results

showed that two black rice cultivars (Asamurasaki, Okunomurasaki) contained three major anthocyanins: cyanidin-3-glucoside, peonidin-3-glucoside and malvidin. Chinakuromai (black) rice additionally contained a fourth anthocyanin, petunidin-3-glucoside. Four red rice cultivars contained only malvidin. The total anthocyanin content varied greatly among black rice cultivars (79.5-473.7 mg/100 g), but was lower in red rice (7.9-34.4 mg/100 g).

Chapter 3 shows total phenolic contents (TPC) of different colored rice strains. TPC of white and green rice (124.8 mg/100 g and 160.0 mg/100 g, respectively) was significantly lower than that of black and red rice. TPC was similar between red (460.3-725.7 mg/100 g) and black (417.1-687.2 mg/100 g) rice, and varied markedly in the same colored rice. Furthermore, TPC appeared to be specific for each cultivar of colored rice rather than being a general property of rice color.

Chapters 4 and 5 describe comparison of antioxidant capacity and TPC by various extracted solvents and test methods. The results showed that antioxidant capacities of different colored rice strains were ranked as follows: red (69.9-130.3 μmol Trolox/g) > black (55.5-64.9 μmol Trolox/g) > green (35.3μmol Trolox/g) > white (21.8 μmol Trolox/g) rice. The antioxidant capacity owned mainly to pericarp and testa, not the endosperm. Besides, the stronger polar extracted solvent (1% HCl methanol) showed the strongest antioxidant capacities and higher total phenolic content among three different extract methods.

Chapter 6 shows expression analysis of five genes involved in anthocyanin biosynthesis; chalchone synthase (CHS). chalchone isomerase (CHI), dehydroflavonol-4-reductase (DFR), leucoanthocyanidin reductase (LAR), and anthocyanidin synthase (ANS) using real time PCR technique. Flavonoid biosynthetic genes have been extensively studied in grape, *Arabidopsis* and *Petunia*, but little research has been conducted into the regulatory pathways in rice. This study showed that the expression of five flavonoid biosynthetic genes, presented in all colored strains studied, including white, black and red rice, differed dramatically depending on the gene, tissue and stage of development; however, little difference was observed during development and in the different tissues of white rice.

Chapters 7 and 8 show the results of expression analysis of five genes of black rice in response to sugar and light. The results of sugar response showed that the expression of CHS, CHI, DFR, LAR and ANS were strongly and dose-dependently induced in response to sucrose (Suc), and different gene occurred in difference of reaction induced, for example, DFR was the stronger than that of the other four genes. However, glucose (Glc) suppressed strongly the transcription of flavonoid biosynthetic genes under the assessed conditions, and treatment with mixture of Glc and Suc also strongly suppressed transcript of flavonoid biosynthetic genes, for example, for DFR transcript level was 200-fold lower than that of control. ANS, CHI, LAR and CHS presented similar DFR results. In brief, in this study, Glc negatively regulated flavonoid biosynthethic gene transcription.

White light induced the transcript of all flavonoid biosynthetic genes, and transcript levels significantly increased depending on the length of exposure to white light, for example, DFR, LAR, ANS and CHI transcript levels were greater after a 24 h exposure than at 16 h. Besides, different genes to sensitive degree of light existed in difference, for example, CHS transcript levels at 16 h were greater than those observed at 24 h. Lastly, differences were observed in the transcript levels of different genes with exposure to white light; for example, DFR was more strongly induced than the other four genes.

In conclusion, the results present this paper will be useful for breeding, food processing, agronomy and plant physiology field in the near future.

Chapter 1: Introduction

With improvement of human life, functional food that is rich in nutrition, good for health, and with an appealing color is becoming increasingly important. Colored rice is a special rice that has a series of pharmacological effects, and plays an important role in nutrition and health ingredients. Black rice is rich in various anthocyanin pigments, such as cyanidin-3-gluctoside, malvidin and peonidin-3-gluctoside, petunidin-3-gluctoside. Red rice also contains anthocyanin pigment although slightly less than black rice. Furthermore, red rice possesses a high content of proanthocyanidin. Anthocyanin and proanthocyanidin are both flavonoids.

1. Flavonoid

1.1. Structure of flavonoid compounds

Flavonoids are secondary metabolites of plants, and are polyphenols, also called Vitamin P. The basic structure of a flavonoid compound is C6-C3-C6 (Guerrero et al., 2012) (Fig.1.1)

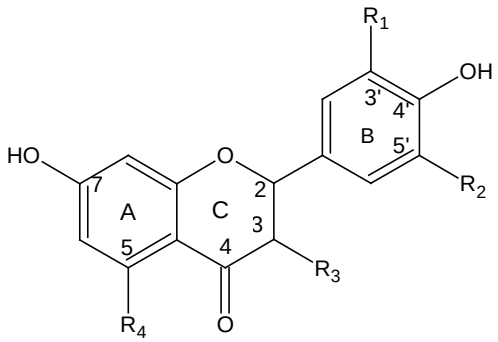


Fig.1.1 Basic structure of flavonoid compounds R_1, R_2, R_3, and R_4 representing radicles that can be modified.

The basic skeleton of flavonoids was formed by a series of compounds with two aromatic rings A and B that are connected with each other through the central carbon chain. According to the oxidant degree of the central three-carbon chain, connection position of the B ring in the C ring, as well as constitutes of three-carbon chain, it was

divided into flavonoid glycosides, flavonols, chalcone, anthocyanin, proanthocyanidin and so on (Martinelli et al., 1986).

1.2. Biosynthetic pathway of flavonoid

The whole biosynthetic pathway of flavonoids has been determined by cloning gene of maize, *Arabidopsis thaliana* and *Antirrhinum majus* (Donner, 1991; Tanaka, 1998; Brenda, 2001; Xiao, 2011;). The synthesis pathway is as follows (Fig.1.2)：

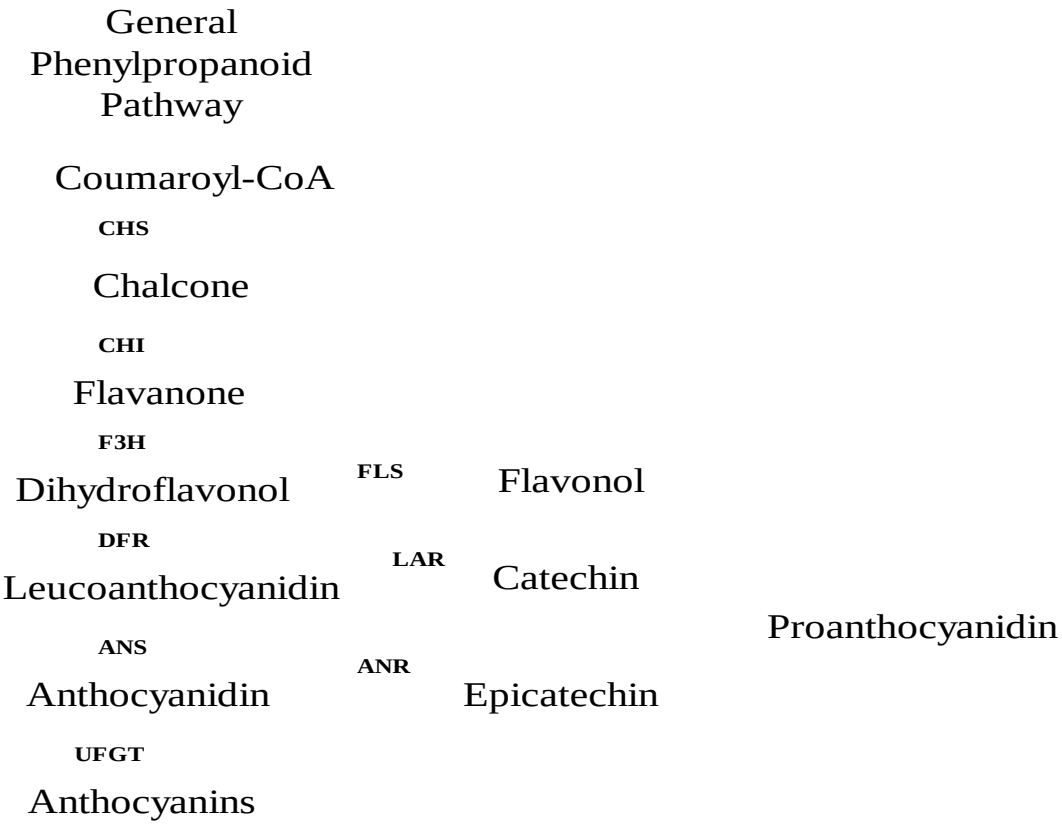


Fig.1.2 Synthetic process of flavonoid

CHS, chalcone synthase; CHI, chalcone isomerase; F3H,flavanone 3 hydroxlyase ;
DFR, dihydroflavonol 4-reductase; LAR, leucoanthocyanidin reductase; ANR, anthocyanidin reductase; ANS, anthocyanidin synthase; FLS, flavonol synthase; UFGT, UDP-glucose flavonoid 3-o-glucosyl transferase.

2. Progress of anthocyanin research

The anthocyanin pigment is a flavonoid. It is ubiquitous throughout the plant kingdom, such as, fruits, flower, seed, leaf and stems.

The research has focused on anthocyanin structure, function, pathway of synthesis, regulating genes identified by cloning maize, petunia and *Arabidopsis thalania* and so on (Mol et al., 1998; weisshaar and Jenkins, 1998; Springob et al., 2003).

2.1. Structure and classification of anthocyanin

Anthocyanin can produce several colors ranging from red, blue and purple to black, and is mainly classified into three species types with a common chemical structure C_6-C_3-$C_{6,}$ and different numbers of OH at different locations. They are pelargonidin, cyanidin, delphinidin and so on (Boase and Davies, 2006; Davies, 2009).

Rice is generally categorized by caryopsis color at harvest time into all kinds of colors, such as red, black, brown and purple because of precipitation of anthocyanin pigments (Han et al., 2004).

2.2. Physiological function of anthocyanin

Anthocyanin is also involved in a wide range of biological functions. For example, it plays a protective role in plant-microbe interactions, and participates in the plant defense response. Recently, some studies have also stressed the involvement of flavonoids in seed, imposed dormancy as well as in seed storage. Furthermore,

flavonoids are receiving increasing interest as health-promoting components for animals and humans. Anthocyanin also defends powerfully against ultraviolet ray. Therefore, the plant can escape damage. These diverse roles can be correlated, at least in part, with the well documented antioxidant properties of phenylpropanoid.

2.3. Factors of anthocyanin affected

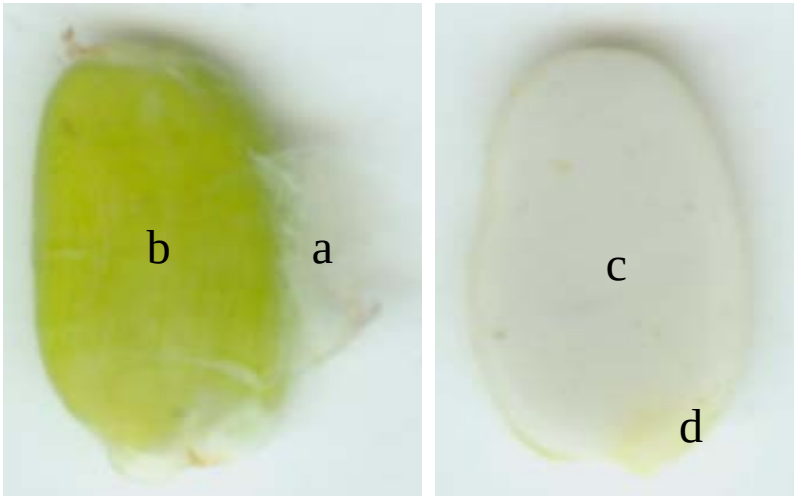
Anthocyanin exists in the fruit, seed capsule, pericarp, and cereal grain, and has a red, purple, blue and black color.

Anthocyanin biological synthesis is affected by genetic factors, environment, nutrients，microelement and mineral. Genetics of anthocyanin is greatly complex. For example, anthocyanin pigment is determined by the main gene, and a few minor genes. Furthermore, it is also affected by environment, such as light: high-intensity light can enhance synthesis of anthocyanin. Shortage of water can also enhance the synthesis of anthocyanin (Donner and Robbins, 1991).

2.4. Expression of anthocyanin pigments in rice

Anthocyanin pigments in rice are expressed in the sheath, seed capsule, and stigma, hull, but was not found to exist in endosperm (Fig.1.3). The most common colored rice strains are black rice, red rice, white rice (common rice), and few green rice (Fig.1.4) (Itani, 2000). There have been a number of studies about the genetic make-up of rice. For example, anthocyanin pigments of black rice have been found to be determined by the main gene and a series of minor genes. The anthocyanin

pigment in red rice was determined by interaction of Rc and Rd gene, and the color in red rice becomes abnormal by the lack of any one, for example, red rice presents reddish-brown spots if anthocyanin pigment controlled by just only Rc. Furthermore, it is colorless if the controlled gene is only Rd (Nagao and Takahashi, 1963).



A B

Fig.1.3 Structure of caryopsis

A: Outside of caryopsis; B: Inside of caryopsis; a: Pericarp; b: Testa; c: Endosperm. d: embryo

Fig.1.4 Four kinds of colored rice strains

A. White rice; B. Green rice; C. Black rice; D. Red rice

3. Aim of research

The purpose of this study was to identify the component of anthocyanin pigments in four kinds of colored rice; red, black, green and common rice (white rice), to analyze why the results differed with the study, using the many colored rice cultivars from different countries and regions in our laboratory. It is possible that the difference in results derived from the difference in genetic makeup. Therefore, I further conducted studies to identify the anthocyanin pigments and to obtain information on the differences occurring with the cultivar. Various studies have been conducted to analyze the antioxidant capacity, and focused mainly on caryopsis. There are few reports on the antioxidant capacity of the endosperm. Furthermore, there are no reports on the antioxidant capacity of green rice. Therefore, we analyzed the

antioxidant capacity of four kinds of colored rice strains in different tissues so that it can provide some evidence for processing of foods, selection of cultivars and so on. In the mean time, it will provide evidence for breeding of cultivars in the near future.

There have been studies on the biosynthetic pathway of anthocyanin pigment using techniques for cloning in maize and petunia. A series of genes that play an important role in biosynthetic pathway of anthocyanin pigment has been identified. Therefore, I analyzed the expression of the genes using real-time PCR in different colored rice strains.

Chapter 2：Identification and qualification of anthocyanin pigment in different colored rice strains

1. Introduction

To date, several reports dealt with the identification of anthocyanin pigments in black rice cultivars, for example, studies showed that the main anthocyanin pigments of black rice were cyanidin-3-glucoside (C3G) and peonidin-3-glucoside (P3G) (Abdel-Aal et al., 2006; Hu et al., 2003; Tian et al., 2005). Yet few reports focused on red rice cultivars, and different researchers lead to different results, for example, Abdel-Aal et al. (2006) reported that C3G was the anthocyanin pigment of red rice, while Kim et al. (2008) reported that red rice did not contain anthocyanin pigments. In this study, we went on identifying and quantifying anthocyanin pigments of three black rice cultivars and four red rice cultivars.

2. Materials and methods

2.1. Materials

Three strains of colored rice: green, red and black rice (eight cultivars in total) and one common rice cultivar (white rice) were selected for the following study. One white rice cultivar (Nakateshinsenbon), four red rice cultivars (Benisarasa, Tsukushiakamochi, Beniroman and Tohboshi), three black rice cultivars (Okunomurasaki, Chinakuromai and Asamurasaki) and one green rice cultivar (Akunemochi) were used. The rice was cultivated in the experimental fields of the

Faculty of Life and Environmental Sciences, Prefectural University of Hiroshima, and harvested in 2009. Seeds were stored at 4°C until use.

2.2. Chemicals

Five authentic anthocyanin standards, C3G, P3G, Pt3G, malvidin and cyanidin were purchased from Takaiwa Phytochemical (Co. Ltd., Chiba, Japan). Other chemicals were purchased from Wako (Hiroshima, Japan), and were of the highest purity available.

2.3. Sample preparation

Randomly selected caryopsis of each colored cultivar was milled with an ultra centrifugal mill (Iwatani, Japan). Milled rice powder (2.0 g) was soaked in 1% HCl methanol, and sequentially extracted for 6 h at room temperature with shaking. The total volume of extract was adjusted to 50 ml with 1% HCl methanol. All extracted samples were stored at -80°C until use.

2.4. Measurement of total anthocyanin content and identification of anthocyanin

Separation of anthocyanins was carried out with an HPLC system equipped with an InertSustain C18 (5 µm, 250 × 4.6 mm ID) column, a VIS 535 nm (LC800 UV) detector (Hitachi, Tokyo, Japan), using an injection volume of 20 µl, and operated at room temperature with a flow rate of 1 ml/min. Elution was carried out using a gradient system with $H_2O/CH_3CN/CH_3OH/HCOOH$ = 40/22.5/22.5/10, v/v/v/v

(solvent A) and H_2O/CHOOH = 90/10, V/V (solvent B) as follows: 7 min, 7% A and 93% B; 35 min, 25% A and 75% B; 10 min, 65% A and 35% B. Five authentic anthocyanin standards, C3G, P3G, Pt3G, malvidin and cyanidin (Tokiwa Phytochemical Co., Ltd., Chiba, Japan), were used for the quantitative analysis of anthocyanin pigments.

The mass spectrograph data were analyzed by the Institute of Chemistry of Chinese Academy of Sciences. The LC system was equipped with a Finnigan Suryor pump and a Suryor autosample injection apparatus. The ion trap mass spectrometer was LCQ Deca XP^{TM} (Finnigan, USA). MS analysis were carried out on ion trap mass spectrometer with an electrospray ionization source that was set to the positive ion mode.

2.5. Statistical analysis

All measurements were carried out three times from the same extract in order to determine reproducibility. Analysis of variance was used to determine difference in TAC. Statistical significance was accepted at $p < 0.05$, determined using a *t*-test.

3. Results and discussion

A number of studies have shown that anthocyanin pigments from black rice were composed mainly of C3G and P3G (Abdel-Aal et al., 2006; Hu et al., 2003; Tian et al., 2005). Our study found that black rice cultivars contained three to four anthocyanins (Fig.2.1). HPLC revealed that the varieties Asamurasaki and Okunomurasaki

possessed three types of anthocyanin pigments, while Chinakuromai had four. Further identification, using authentic anthocyanin standards and mass spectrograph, showed that Asamurasaki and Okunomurasaki contained the macromolecular anthocyanins P3G and C3G, and the micromolecular anthocyanin malvidin, while Chinakuromai contained the macromolecular anthocyanin Pt3G, in addition to P3G, C3G, and malvidin. Ryu, Park and Ho (1998) reported that different black rice varieties contained different ratios of anthocyanins; for example, Suwon 415 contained 95.3% C3G, whereas Suwon 425 and Heugjinmi showed a relatively larger amount of P3G. The results of our quantitative analysis (Table 2.1) showed significant differences between the various cultivars. For example, C3G was the most abundant anthocyanin in Asamurasaki and Okunomurasaki rice (52% and 60% of the total content, respectively), with lower contents of P3G (30% and 12%, respectively) and malvidin (17% and 28%, respectively) in these cultivars. Although Pt3G has rarely been reported as an important anthocyanin pigment in rice, we found Pt3G to be the predominant anthocyanin in the Chinakuromai cultivar (168.3 mg/100 g), comprising almost half of the total anthocyanin content. In contrast, C3G was observed at a lower level (30.8 mg/100 g, 9% of total anthocyanin content) in this cultivar. Malvidin has rarely been reported in black rice. However, it was detected in black rice in our study although its content was low.

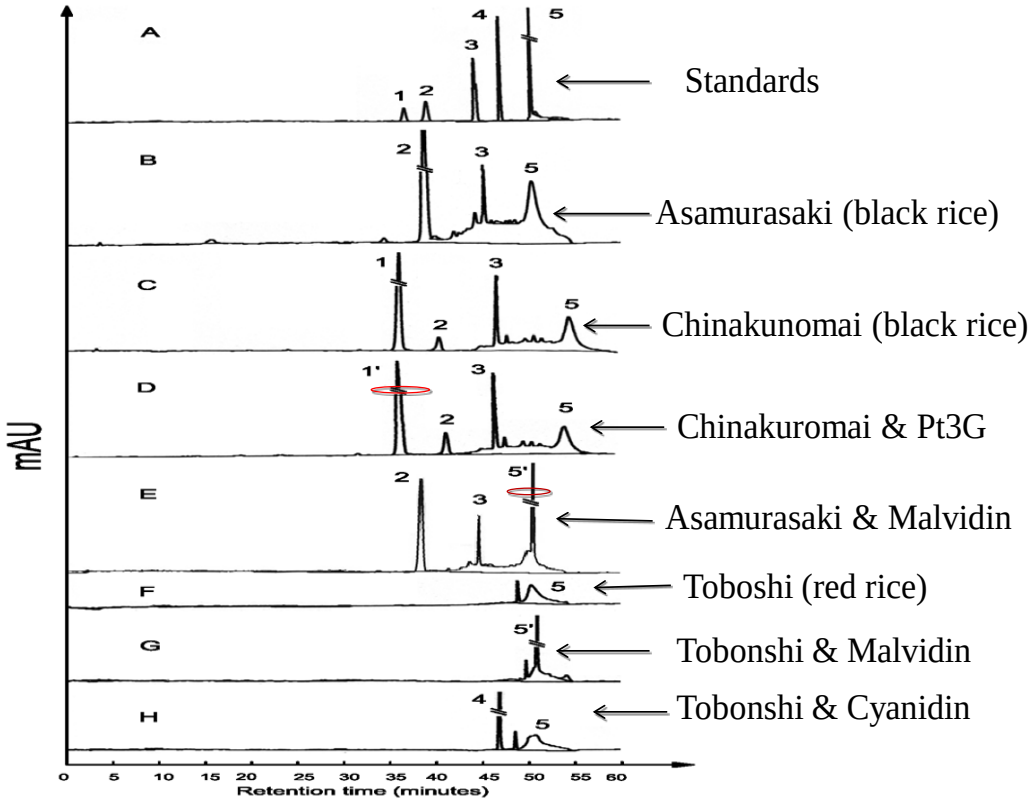


Fig.2.1 Typical chromatogram of standard anthocyanin pigments and anthocyanins of two black and one red rice cultivars

A, Chromatogram of a mixture of five kinds of standard anthocyanin pigments. Peaks: 1, petunidin-3-glucoside; 2, cyanidin-3-glucoside; 3, peonidin-3-glucoside; 4, cyanidin; 5, malvidin; 1', peak of mixture of Pt3G standard and Chinakuromai; 5', peak of mixture of malvidin standard and black rice Asamurasaki or red rice. B, Chromatogram of black rice Asamurasaki. C, Chromatogram of black rice Chinakuromai. D, Chromatogram of mixture of Chinakuromai and Pt3G standard. E, Chromatogram of mixture of Asamurasaki and malvidin standard. F, Chromatogram of red rice. G, Chromatogram of mixture of red rice and malvidin standard. H, Chromatogram of mixture of red rice and cyanidin standard

Malvidin was the only anthocyanin pigment detected (using HPLC) in the four cultivars of red rice (Fig.2.1). The malvidin contents were as follows: Tohboshi, 16.9 mg/100 g; Benisarasa, 24.3 mg/100 g; Tsukushiakamochi 7.9 mg/100 g; and Beniroman, 34.4 mg/100 g (Table 2.1). Some reports showed that red rice does not

contain any anthocyanin pigments (Oki et al., 2002; Kim et al., 2008), while others reported C3G as the anthocyanin pigment of red rice (Abdel-Aal et al., 2006; Yao et al., 2010). The inconsistencies between these observations regarding anthocyanin pigments in red rice may be due to genetic factors, leading to the production of different anthocyanin pigments.

Table 2.1 Anthocyanin content and ratios in four different colored rice groups

Cultivars	Anthocyanin (mg/100 g)[A]				TAC (mg/100 g)
	C3G	P3G	Pt3G	Malvidin	
White rice					
Nakateshinsenbon	nd	nd	nd	nd	nd
Green rice					
Akunemochi	nd	nd	nd	nd	nd
Red rice					
Tohboshi	nd	nd	nd	16.9±5.4	16.9
Benisarasa	nd	nd	nd	24.3±1.8	24.3
Tsukushiakamochi	nd	nd	nd	7.9±1.7	7.9
Beniroman	nd	nd	nd	34.4±8.3	34.4
Black rice					
Chinakuromai	30.8±1.2 (9%)	101.0±7.8 (30%)	168.3±9.1 (49%)	40.9±2.7 (12%)	341.0
Asamurasaki	249.6±2.0 (52%)	142.8±25.6 (30%)	nd	81.2±19.5 (17%)	473.7
Okunomurasaki	48.0±0.2 (60%)	9.4±2.8 (12%)	nd	22.1±5.0 (28%)	79.5

[A]Values are expressed as means ± SD (n = 3). TAC, total anthocyanin content. nd, no detected.

Our results showed that black rice was rich in macromolecular anthocyanins, and also contained the micromolecular anthocyanin, malvidin. In contrast, red rice contained only malvidin, and was characterized by a low TAC content.

4. Conclusions

Black rice cultivars contain different anthocyanin pigments because of difference of genetic background. There are rather higher content in all cultivars, and occurred to significant difference among different cultivars. In contrast, red rice cultivars only contain micromolecular malvidin, and contents were rather low in all cultivars. Therefore, this study will provide proof for breeding of cultivars.

Chapter 3: Measurement of phenolic contents in different colored rice strains

1. Introduction

Few report existed with respect to phenol in colored rice strain although colored rice possess rich phenol, and different researches lead to different results, for example, Yao et al. (2010) reported that black rice possessed the highest total phenolic content (TPC) among three strains, red, black, and purple rice. While, Sompong et al. (2011) recently reported that TPC significantly differed between cultivars of the same colored rice rather than between different colored rice. Therefore, we continued to study phenol content in different colored rice strains in view to inconsistent results reported.

2. Materials and method

2.1. Materials

Materials were the same as shown in Chapter 2.

2.2. Chemicals

Folin-Ciocalteu's reagent was obtained from Sigma Chemical (Co. USA). Gallic acid monohydrate and other chemicals were purchased from Wako (Hiroshima, Japan), and were of the highest purity available.

2.3. Sample extraction

Randomly selected caryopsis of each colored cultivar was milled with an ultra centrifugal mill (Iwatani, Japan). Milled rice powder (2.0 g) was soaked in 1% HCl methanol, and sequentially extracted for 6 h at room temperature with shaking. The total volume of extract was adjusted to 50 ml with 1% HCl methanol. All extracted samples were stored at -80°C until use.

2.4. Measurement of total phenolic content (TPC)

TPC was determined by the Folin-Ciocalteu method (Zhang, 2010) with slight modification. In brief, to generate a standard curve, 0.05 g gallic acid monohydrate was dissolved in 80% methanol and adjusted to 50 ml with 80% methanol. Aliquots (0.025, 0.05, 0.10, 0.20, 0.40 and 0.60 ml) of the 50 ml gallic acid monohydrate solution were transferred to cuvettes, and 1.25 ml of Folin-Ciocalteu's reagent and 3.75 ml of 20% Na_2CO_3 solution were added to each cuvette. Lastly, the solution was incubated at 30°C for 2 h. A solution without gallic acid monohydrate was used as the control. TPC was calculated by absorbance at 760 nm using an UV spectrophotometer (DU 530, BECKMAN, USA).

2.5. Statistical analysis

All measurements were carried out three times from the same extract in order to determine reproducibility. Analysis of variance was used to determine differences in TPC. Statistical significance was accepted at $p < 0.05$, determined using a *t*-test.

3. Results and discussion

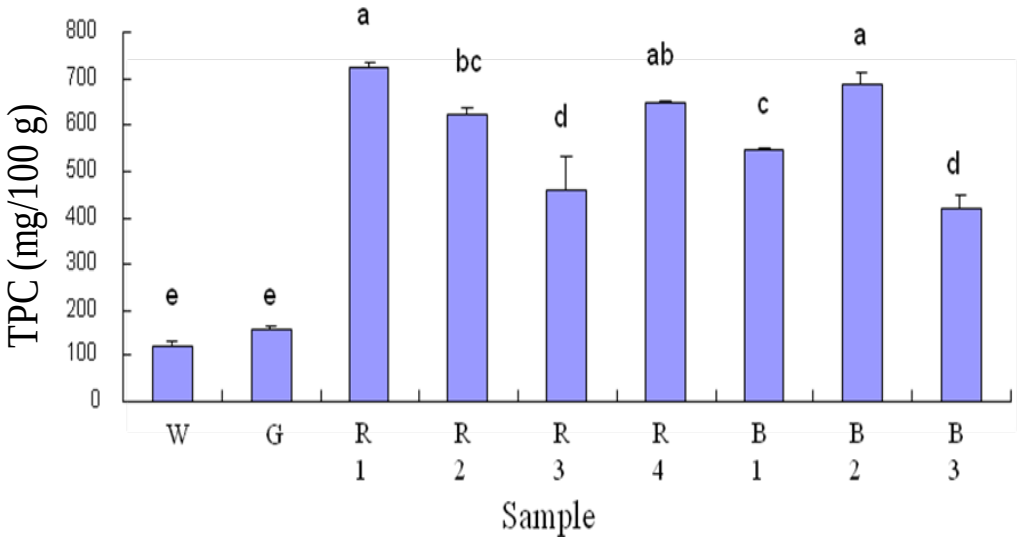


Fig.3.1 Total phenolic content of different colored rice cultivars.

W: white rice (Nakateshinsenbon); G: green rice (Akunemochi); R: red rice; B: black rice. R1, Tohboshi; R2: Benisarasa; R3: Tsukushiakamochi; R4: Beniroman; B1: Chinakuromai; B2: Asamurasaki; B3: Okunomurasaki. Data are expressed as mean values (n = 3). Identical superscript letters denote TPCs that are not significantly different at $p < 0.05$.

Total phenolic content (TPC) of samples, which were prepared in 1% HCl methanol, was evaluated by UV spectrophotometry (Fig.3.1). The results showed that TPC of white rice and green rice (124.8 mg/100 g and 160.0 mg/100 g, respectively) were significantly lower than black and red rice. Yao et al. (2010) reported that black rice (8.6 g/100 g) possessed the highest TPC among three strains (red, purple and black rice), the TPC of black rice was 86-fold greater than that of red rice (0.1 g/100 g). Sompong et al. (2011) recently reported that TPC (using ten red rice cultivars and three black rice cultivars) significantly differed between cultivars of the same colored

rice rather than between different colored rice. Moreover, the highest TPC was found in the red Thai rice (691 mg/100 g). Similar to the results of Sompong, there were no significant differences between black rice cultivars (417.1-687.2 mg/100 g) and red rice cultivars (460.3-725.7 mg/100 g), as shown in Fig.3.1. The highest TPC content was found in red rice (Tohboshi; 725.7 mg/100 g). We further observed that TPC varied markedly in the same colored rice. TPC appears to be specific for each cultivar of colored rice rather than being a general property of rice color.

4. Conclusion

TPC showed the rather low in green rice and white rice. In contrast, black rice and red rice possessed the higher content about TPC, and appeared to significant difference in the same colored rice rather than between different colored rice strains. It provides a proof for analysis of food nutrition components.

Chapter 4: Antioxidant analysis in different colored rice strains

1. Introduction

Rice (*Oryza sativa* L.) is consumed as a staple food by over half of the world's population, and the main cultivation regions include South Asia, China, Korea, Thailand, Japan and so on. Rice is generally categorized by color, which is determined by the composition of anthocyanin pigments that are ubiquitous throughout the plant kingdom, into green, red, black, as well as common rice (white rice). Anthocyanin pigments are flavonoid and play an important role in reducing the risk of oxidative damage, cancer, and cardiovascular disease (Acquaviva et al., 2003; Harborne, 1997; Harborne & Williams, 2000; Lazze et al., 2003; Osawa et al., 1992; Russo et al., 2005).

A considerable number of studies on the antioxidant activity of black rice have been conducted in the last three decades (Hiemori et al., 2009; Hu et al., 2003; Ling et al., 2001; Laokuldilok et al., 2011; Toyokuni et al., 2002; Vsteen, 1983). However, few reports exist with respect to red rice and no study has dealt with the antioxidant analysis of green rice. Moreover, scarce information is available regarding the comparative analysis of antioxidant activity among different colored rice strains. In this study, we compared antioxidant activity, determined using the oxygen radical absorbing capacity (ORAC) assay, among colored rice strains, and among cultivars in the same colored strain. To date, most studies have focused on the antioxidant

capacity of the bran and caryopsis in black rice (Zhang et al., 2010; Laokuldilok et al., 2011). Although the endosperm is considered as a highly nutritious part of the grain, there has been scant antioxidant analysis of the endosperm of colored rice. Therefore, this study determined the antioxidant activity of the endosperm from different colored rice strains. Besides, we subsequently evaluated ORAC using authentic standards of four kinds of anthocyanin in view to know antioxidant capacity of anthocyanin pigments.

Though colored rice is rich in phenolic acids, few studies have been reported on whether phenolic content affects antioxidant capacity (Zhang et al., 2010). We assessed the effect of phenolic and anthocyanin contents on antioxidant capacity in the caryopsis of colored rice.

2. Materials and methods

2.1. Materials

Materials were the same as shown in Chapter 2.

2.2. Chemicals

2,2-azobis 2-amidino-propane dihydrochloride (AAPH), Folin-Ciocalteu's reagent, 6-hydroxy-2,5,7,8-tetramethylchroman-2-carbonsaure (Trolox) were obtained from Sigma Chemical (Co. USA). Other chemicals were purchased from Wako (Hiroshima, Japan), and were of the highest purity available.

2.3. Sample preparation

Randomly selected caryopsis of each colored cultivar was milled with an ultra centrifugal mill (Iwatani, Japan). Milled rice powder (2.0 g) was soaked in 1% HCl methanol, and sequentially extracted for 6 h at room temperature with shaking. The total volume of extract was adjusted to 50 ml with 1% HCl methanol. Samples extracted with 1% HCl methanol were used for ORAC assay. Endosperm samples of the colored rice cultivars, polished to 90% by a rice polisher (Panasonic, Tokyo, Japan), were analyzed using the ORAC assay, and the extraction method employed was the same as for caryopsis. All extracted samples were stored at -80°C until use.

2.4. Oxygen radical absorbing capacity (ORAC) assay

Assays determining antioxidant capacity were carried out on a Thermo Labsystems Fluoroskan Ascent FL plate reader (Sigma Chemical Co. USA). The temperature of the incubator was set at 37°C. The procedure employed was based on the method of Wu et al. (2004) and Huang et al. (2002). In brief, AAPH was used as the peroxyl radical generator, Trolox as the standard, and fluorescein as the fluorescent probe. With the use of appropriate filters, an excitation wavelength of 485 nm was selected, and fluorescence emission at 520 nm was measured every 2 min over a 90-min period. The measurements were taken in triplicate. The final ORAC results were standardized to Trolox. The fluorescence analysis system automatically generated Trolox standard curves using net fluorescence (background-subtracted values). The relative Trolox equivalent ORAC value was calculated as follows:

relative ORAC value = (AUC_{sample} − AUC_{blank}) micromoles of Trolox equivalents per gram of dry weight / (AUC_{Trolox} − AUC_{blank}) micromoles of Trolox equivalents per gram of dry weight. The data were analyzed using a Microsoft Excel macro program (Microsoft, Roselle, IL, USA.)

2.5. Statistical analysis

All measurements were carried out three times from the same extract in order to determine reproducibility. Analysis of variance was used to determine differences in antioxidant activity. Statistical significance was accepted at $p < 0.05$ using a *t*-test.

3. Results and discussion

3.1. Antioxidant capacity of the caryopsis

The antioxidant capacity of one white, one green, four red, and three black rice cultivars was evaluated using the ORAC assay (Table 4.1). The results showed that the antioxidant capacity of rice followed the rank order: red rice cultivars (69.9-130.3 μmol Trolox/g) > black rice cultivars (55.5-64.9 μmol Trolox/g) > green rice (35.3 μmol Trolox/g) > white rice (21.8 μmol Trolox/g). Furthermore, significant differences were observed between the antioxidant capacities of individual cultivars within red colored rice. For example, the red rice cultivar Tsukushiakamochi showed two-fold greater antioxidant capacity than Tohboshi. In contrast, the value did not markedly vary among the three black rice cultivars. In a comparison of red, black, and

purple rice cultivars, Yao et al. (2010) reported that the 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging activity of black rice was greater than that of red rice; similar results were reported by Laokuldilo et al. (2011). In contrast, Oki et al. (2002) reported that the DPPH radical scavenging activity of red-hulled rice was greater than that of black and white-hulled rice. However, our data showed that the antioxidant capacities of two of the four red rice cultivars were significantly greater than those of the black rice cultivars, as determined by the ORAC assay. It is possible that the genetic background of the rice, as well as environment factors during cultivation, led to the observed differences in antioxidant capacity.

Table 4.1 Oxygen radical absorbing capacity (ORAC) of rice caryopsis and endosperm[A]

Cultivars	μmol Trolox/g		Ratio of endosperm to caryopsis (%)
	ORAC values of caryopsis	ORAC values of endosperm	
White rice			
Nakateshinsenbon	21.8±1.5^{h}	8.5±0.9^{e}	39
Green rice			
Akunemochi	35.3±4.3^{fgh}	8.0±1.4^{e}	14
Red rice			
Tohboshi	69.9±11.1^{cd}	20.7±3.3^{b}	30
Benisarasa	91.0±14.6^{bc}	14.9±1.4^{bcd}	16
Tsukushiakamochi	130.3±17.8^{a}	30.6±3.4^{a}	23
Beniroman	100.4±10.7^{b}	10.9±2.1^{de}	11
Black rice			
Chinakuromai	64.0±8.3^{cdef}	20.5±3.2^{bc}	32
Asamurasaki	64.9±6.2^{cde}	6.5±0.5^{e}	10
Okunomurasaki	55.5±5.6^{fg}	5.1±0.7^{e}	9

[A]Values are expressed as means ± SD (n = 3). Values within each column with the identical superscript letter are not significantly different at $P< 0.05$.

3.2. Antioxidant capacity of the endosperm

The antioxidant capacity of the endosperm of colored rice, which was prepared as for caryopsis, was evaluated by the ORAC assay (Table 4.1). The results showed that the antioxidant capacity of endosperm was generally rather low compared with that of caryopsis; for example, the antioxidant capacity of Tsukushiakamochi endosperm was 30.6 μmol Trolox/g, four-fold lower than its caryopsis (130.3 μmol Trolox/g). Furthermore, the antioxidant capacity of endosperm did not vary as greatly as for caryopsis (Table 4.1); for example, the antioxidant capacity of white rice (8.5 μmol Trolox/g) and green rice (8.0 μmol Trolox/g) was similar to that of two black rice cultivars, Okunomurasaki (5.1 μmol Trolox/g) and Asamurasaki (6.5 μmol Trolox/g), and the red rice cultivar Beniroman (10.9 μmol Trolox/g). However, significant differences among different colored rice strains were only observed for the red rice cultivar Tsukushiakamochi (30.6 μmol Trolox/g) and the black rice cultivar Chinakuromai (20.5 μmol Trolox/g). The antioxidant capacities of Nakateshinsenbon, Chinakuromai, and Tohboshi endosperm, relative to caryopsis, were greater than those of the other cultivars, with values 30% higher in the endosperm than in the respective caryopsis. However, the ratio of antioxidant capacity of endosperm to caryopsis for the other cultivars was rather low. It is possible that the endosperm contains a different ratio of polyphenols or other compound(s) that affect the antioxidant capacity. It thus appears that the antioxidant capacity of rice was mainly determined by the seed capsule, at least for the majority of cultivars.

3.3. Antioxidant activity of authentic anthocyanins

Antioxidant activity of four kinds of authentic anthocyanins, determined using the ORAC assay, followed the rank order: P3G (42.4 µmol Trolox/100 mg) > malvidin (41.6 µmol Trolox/100 mg) > C3G (27.8 µmol Trolox/100 mg) > Pt3G (22.9 µmol Trolox/100 mg) (Fig.4.1). Results indicate that anthocyanin pigments do possess antioxidant capacity.

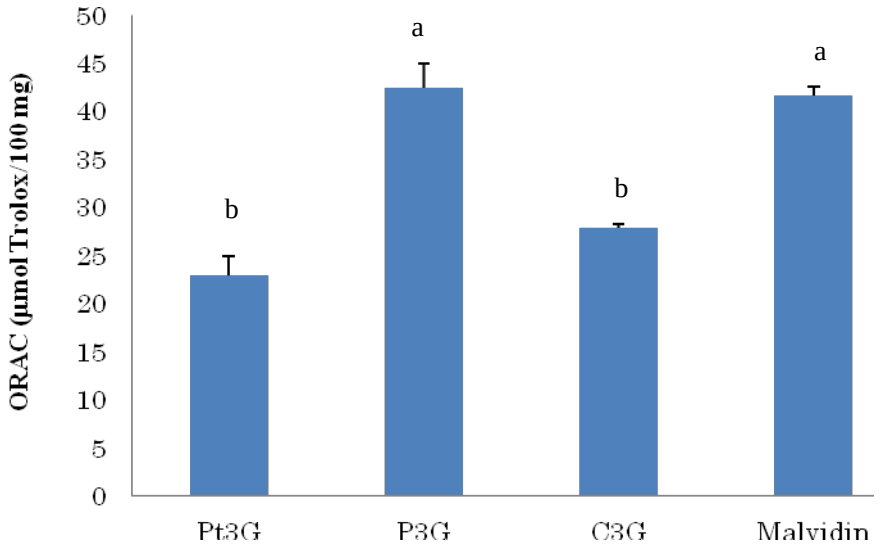


Fig.4.1 Evaluation of the antioxidant capacities of four standard anthocyanins by ORAC assay.

Data are expressed as mean values (n = 3). Identical superscript letters denote ORACs that are not significantly different at $p < 0.05$. Pt3G: Petunidin-3-galactoside; P3G: peonidin-3-glucoside; C3G: cyanidin-3-glucoside.

3.4. Antioxidant capacity of anthocyanin pigments of colored rice

Brown et al. (2005) reported that anthocyanins were the main contributors to TPC and to antioxidant activity. Our results simply do not support this claim. The data in Table 4.2 show that the contribution of TAC to antioxidant capacity was notably low

in the caryopsis. In red rice cultivars, the TAC contributed only 0.03-0.1% to the total antioxidant capacity of the caryopsis. Similarly, in black rice cultivars, TAC contributed only 0.5-2.5% to the antioxidant capacity of the caryopsis. For example, in Asamurasaki (black rice), the calculated ORAC value for TAC was only 1.64 μmol Trolox/g, whereas the total caryopsis value was 64.85 μmol Trolox/g. Therefore, it appears that in the caryopsis, the other phenolic compounds other than anthocyanins are the main contributors to ORAC. Rice-Evans, Miller and Paganga (1997) reported that polyphenols contribute to free-radical scavenging capacity *in vivo* and *in vitro* due to their ideal antioxidant structure. There are many types of polyphenols, such as ferulic acid, ρ-coumaric acid, vanillic acid, ρ-hydroxybenzoic acid, several kinds of anthocyanin pigments and so on. Moreover, different rice cultivars contain different ratios and types of polyphenols. For example, Sompong et al. (2011) found that the most abundant phenolic acid found in red rice varieties was ferulic acid, followed by ρ-coumaric and vanillic acids. The most common type among black rice varieties was ferulic acid, followed by vanillic and ρ-coumaric acids. Therefore, the high antioxidant capacity of red rice compared to black rice might be due to differences in the ratios of the contained polyphenols, as phenolics differ in their antioxidant capacity. This would underlie the observed differences in the antioxidant capacities of rice strains. For example, Abdel-Aal and Hucl (1999) reported that the antioxidant capacity of γ-oryzanol was almost ten times higher than that of tocopherols. Furthermore, the antioxidant capacity of α-tocopherol and γ-oryzanol was also reported (Laokuldilok et al., 2011). Besides, 2-arylbenzofuran showed strong

antioxidant activity among black rice (Han et al., 2004). However, further studies are needed to assess the antioxidant capacities of different phenolic compounds and determine how the structure of these affects their antioxidant capacity.

In our experiments, anthocyanin pigments were found to have a low antioxidant capacity. It remains possible that anthocyanins might have interfered with ORAC measurements, resulting in the underestimation of their antioxidant activity.

Table 4.2 Oxygen radical absorbing capacity (ORAC) of four kinds of anthocyanin pigments and caryopsis anthocyanin contribution to ORAC

Cultivars	ORAC of anthocyanin pigments (μmol Trolox/g)				Estimated ORAC	
	C3G	P3G	Pt3G	malvidin	value of TAC	ratio[a]
White rice						
Nakateshinsenbon	nd	nd	nd	nd		
Green rice						
Akunemochi	nd	nd	nd	nd		
Red rice						
Tohboshi	nd	nd	nd	0.070	0.070	0.1%
Benisarasa	nd	nd	nd	0.100	0.100	0.1%
Tsukushiakamochi	nd	nd	nd	0.003	0.003	0.03%
Beniroman	nd	nd	nd	0.143	0.143	0.1%
Black rice						
Chinakuromai	0.09	0.43	0.39	0.17	1.08	1.7%
Asamurasaki	0.69	0.61	nd	0.34	1.64	2.5%
Okunomurasaki	0.13	0.04	nd	0.09	0.26	0.5%

[a]contribution of ORAC of TAC to total ORAC in caryopsis (see table 4.1).

4. Conclusion

In sum, colored rice strains possessed rather strong antioxidant capacity than white rice, and red rice was the highest among colored rice strains, at least in our

study. Besides, antioxidant capacity focused mainly on seed capsule rather than endosperm. Therefore, this study provides proofs for health food and living.

Chapter 5: Comparison of antioxidant capacities and phenolic content among colored rice strains using various solvents and test methods

1. Introduction

Rice (*Oryza sativa* L.) is consumed as a staple food by over half of the world's population, the most common type being white rice (Bhattacharjee et al., 2002). Other types of rice come in various colors, the most common of which are black and red rice, and a few green rice. Black and red rice have been considerably studied for their functional activities, such as antioxidant capacity, content of protein, vitamins and minerals, effectiveness in reducing cholesterol levels in the human body, and inhibitory effects of extracts of pigmented rice bran on *in vitro* allergic reactions (Itani et al., 2002; Suzuki et al., 2004; Choi et al., 2007; Lee et al., 2008; Chen et al., 2012).

Isolation of antioxidants from plant materials can be carried out using different techniques and solvents because of the natural diversity of these compounds and often unique distribution of these compounds in the plant (Antolovich et al., 2000; Sultana et al., 2009). Extraction using solvents, such as acetone, methanol, and ethanol, as well as aqueous mixtures, is the most frequently used technique for analyzing antioxidants in fruits, vegetables, grains, medicinal plants and agro-wastes (Bonoli et al., 2004; Chatha et al., 2006; Sultana et al., 2009). Considerable research has been carried out by analyzing the antioxidant capacity in colored rice using 1% HCl methanol for extraction (Ling et al., 2001; Hu et al., 2003; Hiemori et al., 2009; Chen et al, 2012). There are very few reports of comparative analysis of the antioxidant

capacity in rice using different solvent extracts and different evaluating antioxidant activity methods. Thus, in this study, we compared the antioxidant capacity using different solvents and two methods for measurement in different colored rice strains.

Methods of analyzing the antioxidant capacity are most commonly divided into two types, the electron transfer reaction assay and the hydrogen atom transfer reaction assay. The Trolox equivalent antioxidant capacity (TEAC) assay, the ferric reducing ability of plasma (FRAP) assay, the 2,2-diphenyl-1-picrylhydrazy (DPPH) and the copper reduction (CUPRC) assay belong to the former type. The oxygen radical absorbing (TRAP) assay and oxygen radical absorbing capacity (ORAC) assay belong to the latter type. The antioxidant capacities of phenolic compounds in green tea, orange juice, vegetable juice and apple juice have been analyzed by DPPH, ORAC and TEAC methods (Tabart et al., 2009). There are few reports that the results of the analysis of antioxidant capacity in rice differ with the method used for analysis. Therefore, in this study we compared the results obtained by the DPPH and ORAC assay methods in different colored rice strains.

A number of researchers have studied the antioxidant capacity using different techniques and solvents in other plants, but there have been few reports on the difference in antioxidant capacity corresponding to light time, temperature and rainfall during plant growth. Currently, there have been no reports about the meteorological environment affected hue of colored rice. Therefore, this study analyzed whether there was a correlation between antioxidant capacity and the hue involved in meteorological environment.

Chen et al. (2012) reported that colored rice contains rather higher phenolic content. There are few reports that different solvents affect extraction of phenolics and whether meteorological environment affected synthesis of phenolics in rice. Therefore, this paper measured phenolic content with different extraction solvents and under different factors of meteorological environment.

2. Materials and methods

2.1. Materials

Two red rice cultivars Tsukushiakamochi and Beninoman, two black rice cultivars Asamurasaki and Okunomurasaki, and one white rice cultivar Koshihikari,were used in this study. The rice was cultivated in the experimental fields of the Faculty of Life and Environmental Sciences, Prefectural University of Hiroshima, and harvested in 2005, 2007 and 2011. Seeds were stored at 5°C until use.

2.2. Chemicals

2, 2-azobis 2-amidino-propane dihydrochloride (AAPH), Folin-Ciocalteu's reagent, 6-hydroxy-2, 5, 7, 8-tetramethylchroman-2-carbonsaure (Trolox), 1,1-diphenyl-2-picvylhydrazyl (DPPH) were obtained from Sigma Chemical Company (Co. USA). MES [2-(N-Morpholino) ethanesulfonic Acid] was purchased from Nacalai Tesque. (Inc. Kyoto, Japan). Gallic acid was from Biomedicals (llc. Illkirch, France). Other chemicals were purchased from Wako (Hiroshima, Japan),

and were of the highest purity available.

2.3. Sample preparation

Randomly selected caryopsis of each cultivar was milled with an ultra centrifugal mill (Iwatani, Japan). Milled rice powder (0.2 g) was soaked in 1% HCl methanol or 80% ethanol or 100% methanol, and sequentially extracted for 24 h at room temperature with shaking. The total volume of extract was adjusted to 10 ml with 1% HCl methanol, 80% ethanol and 100 % methanol, respectively. Samples extracted with 1% HCl methanol, 100% methanol and 80 % ethanol were used for measurement of phenolic content, DPPH assay, and an aqueous solution with 1% HCl methanol was also used for the oxygen radical absorbing capacity (ORAC) assay. All extract samples were stored at -20°C until use.

2.4. Statistical method of meteorological environment factors

Statistical rain content, temperature and sunshine time were during 30 days from flowering to harvest of rice (Table 5.1).

Table 5.1 Climate index from after flowering to harvested [a]

Cultivars	Total sunshine (hour)			Daily temperature (mean ℃)			Total rainful (mm)		
	2005	2007	2011	2005	2007	2011	2005	2007	2011
White rice									
Koshihikuri	111.9	159	127.8	22.22	23.82	24.52	165	141	251
Red rice									
Tsukushiakamochi	120.1	155.2	157.6	23.08	22.60	21.97	185	122	154
Beniroman	127.9	147.7	160.6	22.07	21.26	21.77	185	134	154
Black rice									
Asamurasaki	108.7	185.5	137.8	24.5	25.02	24.84	129	125	163
Okunomurasaki	108.7	153.6	137.8	24.5	23.82	24.84	129	146	163

[a] : climate index during 30 days from flowering to harvest of rice.

2.5. DPPH method

The anti-oxidative activity on DPPH radical was estimated according to the anti-oxidative mechanism using a spectrophotometer (Oki. et al. 2002). Briefly, the sample solution (150 μl), 100% ethanol (50 μl), and a 2-morpholinoethanesulfonic acid (MES) buffer (PH 6.0, 0.2 M, 50 μl) and 20% ethanol 50 μl were pipetted into a 96-hole microplate. The reaction was initiated by adding 50 μl of 800 μm DPPH in ethanol. The reaction mixture was shaken with a mechanical shaker and left to stand at room temperature for 20 min in the dark. The solution was measured by spectrophotometry at 510 nm (CS9300PC, using a dual-wavelength flying spot scanning densitometer, Shimadzu Co., Ltd., Kyoto, Japan). The DPPH radical-scavenging activity was estimated from the decrease of absorbance at 510 nm and expressed as Trolox equivalents milliliter of sample solution by using a standard

Trolox curve. All tests and analyses were run in quadruplicate and averaged. Results were tested by one-way analysis of variance using the Statistical Analysis System software package *t*-test.

2.6. ORAC method

Assays determining antioxidant capacity were carried out on a Thermo Labsystems Fluoroskan Ascent FL plate reader (Sigma Chemical Co.). The temperature of the incubator was set at 37°C. The procedure employed was based on the method of Wu et al. (2004). In brief, AAPH was used as the peroxyl radical generator, Trolox as the standard, and fluorescein as the fluorescent probe. With the use of appropriate filters, an excitation wavelength of 485 nm was selected, and fluorescence emission at 520 nm was measured every 2 min over a 90-min period. The measurements were taken in triplicate. The final ORAC results were standardized to Trolox. The fluorescence analysis system automatically generated Trolox standard curves using net fluorescence (background-subtracted values). The relative Trolox equivalent ORAC value was calculated as follows: relative ORAC value = (AUC_{sample} – AUC_{blank}) micromoles of Trolox equivalents per gram of dry weight / (AUC_{Trolox} – AUC_{blank}) micromoles of Trolox equivalents per gram of dry weight. The data were analyzed using a Microsoft Excel macro program (Microsoft, Roselle, IL, USA.).

2.7. Measurement of total phenolic content (TPC)

TPC was determined by the Folin-Ciocalteu method of Zhao et al. (2006) with

slight modification. In brief, to generate a standard curve, 0.05 g gallic acid monohydrate was dissolved in 80% methanol and adjusted to 50 ml with 80% methanol. To aliquots (0.025, 0.05, 0.10, 0.20, and 0.30 ml) of the 50 ml gallic acid monohydrate solution transferred to cuvettes was added 2.5 ml of 10-fold diluted Folin-Ciocalteu's reagent and after allowed to react for 5 min, 2 ml of 75 g/L Na_2CO_3 solution was added to each cuvette, and the final volume was made up to 10 ml with deionied water. Then, the solution was incubated at 37°C for 1 h. A solution without gallic acid monohydrate was used as the control. TPC was calculated by absorbance at 760 nm using a UV spectrophotometer Jasco Ubest-30 (Tokyo, Japan).

2.8. Color measurements

Color was determined by Mc Guire' s method, 1992. The five cultivars were put in plastic bags (0.04 mm thick), and the Hunter L*, a*, and b* values of reflected color were measured using a colorimeter, (CR-200, Minolta Co. Ltd., Japan) with ten repetitions.

3. Results and discussion

3.1. Color of different colored rice strains

Two black rice cultivars, two red rice cultivars and one white rice cultivar were measured using a colorimeter (Table 5.2). The results showed that L*(lightness) value in black rice cultivars ranged from 17.0 to 18.4, that in red rice ranged from 34.6 to 44.5, and that in white rice ranged from 62.6 to 63.6. White rice had the highest L*

value, followed by red rice and black rice. The a*(redness) value in black rice cultivars ranged from 4.1-5.3, that in red rice cultivars ranged from 13.2-19.1, and that in white rice ranged from 4.4-4.5. Red rice had the highest a* value, followed by

Table 5.2 Hunters reflected color values

Cultivars		Hunters reflected color (mean, satandard deviation)			
		L*	a*	b*	H (b/a)
White rice					
Koshihikari	2005	62.7+2.7	4.4+0.6	24.8+1.0	5.6
	2007	63.6+2.1	4.41+0.7	25.1+1.6	5.7
	2011	62.6+2.3	4.5+0.5	24.6+0.8	5.5
Red rice					
Tsukushiakamochi	2005	42.0+2.4	19.1+0.7	29.0+0.7	1.5
	2007	41.2+3.1	17.2+0.9	27.7+1.4	1.6
	2011	44.5+3.0	14.4+1.5	27.4+1.3	1.9
Beniroman	2005	34.7+1.5	13.5+1.4	19.8+1.7	1.5
	2007	34.6+1.8	15.5+0.8	20.9+0.9	1.4
	2011	38.1+2.8	13.2+0.7	21.7+1.1	1.6
Black rice					
Asamurasaki	2005	17.2+2.4	4.1+1.6	1.1+0.7	0.3
	2007	17.9+2.5	4.1+1.6	1.3+0.5	0.3
	2011	18.3+2.1	4.1+1.7	2.0+2.48	0.5
Okunomurasaki	2005	18.2+2.1	5.3+1.4	2.8+1.6	0.5
	2007	17.0+2.1	3.2+0.6	1.0+0.5	0.3
	2011	18.4+2.3	4.8+1.1	2.4+1.3	0.5

Each value is the mean ± standard deviation of ten times. L* = lightness, a* = bluish-green/red-purple hue component, b* = yellow/blue hue component, H (from arctangent b*/a*) = hue angle.

black rice and white rice. The b*(yellowness) value in black rice cultivars ranged from 1.0-2.8, that in red rice cultivars ranged from 19.8-29.0, and that in white rice ranged from 24.6-25.1. Red rice had the highest b* value, followed by white rice and black rice. Furthermore, there were significant differences among red rice and black

rice strains, but little difference from white rice, either in L*, a* or b* value. According to factors of meteorological environment (Table 5.1), the results showed that the hue of colored rice was not correlated with sun shine, rainfall or temperature; for example, there was no significant difference in Asamurasaki between 2005 (sunshine 108.7 hours, L* 17.2) and 2007 (sunshine 185.5 hours, L* 17.9). It is reasonable to assume that the hue angle of colored rice is determined mainly by the genetic background of the rice rather than meteorological environment.

3.2. Comparison of antioxidant capacity in different colored rice strains using different extraction methods

The results showed that the antioxidant capacity of rice differed significantly with the color of rice strains, and was in the rank order: red rice cultivars (1505.8-2065.8 μmol Trolox/100 mg) > black rice cultivars (713.7-1587.4 μmol Trolox/100 mg) > white rice (23.9-92.5 μmol Trolox/100 mg) (Table 5.3). In a study comparing red, black, and purple rice cultivars, Yao et al. (2010) found that the 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging activity of black rice was greater than that of red rice. Similar results were reported by Laokuldilo et al. (2011). In contrast, this result is in accordance with those reported by Oki et al. (2002) and Chen et al (2012), in which the DPPH radical scavenging activity of red-hulled rice was greater than that of either black rice or white-hulled rice. Furthermore, significant differences were observed with the solvent used for extraction in the same cultivar, and 1% HCl methanol exhibited the highest DPPH scavenging activity. The value

obtained using 100% methanol was the lowest for all cultivars examined except for Beniroman (red rice), which showed no significant difference between 80% ethanol and 100% methanol; for example, the value obtained by the DPPH assay for Tsukushiakamochi (red rice) with 1% HCl methanol was 2065.8 μmol Trolox/100 mg, and that obtained with 80% ethanol was 1562.3 μmol Trolox/100 mg, that of 100% methanol was only 1505.8 μmol Trolox/100 mg. Similar to the results obtained using red rice, DPPH values for Asamurasaki (black rice) among three different extraction solvents were significantly different; for example, 1% HCl methanol (1383.8 μmol Trolox/100 mg) was markedly stronger than 80% ethanol (955.9 μmol Trolox/100 mg), or 100% methanol (748.3 μmol Trolox/100 mg). This result was partly in accordance with the report of Anwar and Przybylski (2012) that the antioxidant capacity obtained with 80% ethanol was stronger than that obtained with 100% methanol, and 100% ethanol in flaxseed. Zhao et al. (2006) reported that the extraction solvent had a significant influence on DPPH scavenging activity evaluation in extraction of barley with acetone, 80% ethanol, and 80% methanol. Furthermore, Zhao et al (2006) reported that the 80% acetone extract contained the highest level of (-)-catechin, ferulic, caffeic, vanillic and ρ– coumaric acids, 80% methanol extract contanined the highest level of (-)-epicatechin and syringic acid contents, and the water extract had the highest levels of protocatechuic and gallic acids. Acetone selectively enhanced the catechin and proanthocyanidin extraction yield by Bonoli et al. (2004). In this study, extraction with 1% HCl methanol showed the strongest for antioxidant capacity, followed by that with 80 % ethanol. That of 100% methanol was

the lowest. It is reasonable to presume that different extraction solvents affect the extraction efficiency of different compounds with different antioxidant capacities because of the difference in chemical structure. However, we assume that extraction with a strong polar solvent may promote the extraction of antioxidant compounds. Therefore, this paper provides evidence validating the method of extracting antioxidant compounds.

Table 5.3 Antioxidant capacity determined by DPPH assay after extraction with different solvents in five rice cultivars planted in 2007

Cultivars	DPPH (μmol Trolox/100 mg)		
	80% Ethanol	100% Methanol	1%HCl Methanol
White rice			
Koshihikari	76.8 ± 1.1^{aC}	23.9 ± 1.2^{bC}	92.5 ± 21.1^{aC}
Red rice			
Tsukushiakamochi	1562.3 ± 285.5^{bA}	1505.8 ± 26.7^{bA}	2065.8 ± 240.3^{aA}
Beniroman	1492.7 ± 75.4^{aA}	1633.5 ± 239.4^{aA}	1740.3 ± 195.3^{aAB}
Black rice			
Asamurasaki	955.9 ± 93.9^{bB}	748.3 ± 57.2^{bB}	1383.8 ± 239.8^{aB}
Okunomurasaki	1023.6 ± 272.8^{bB}	713.7 ± 95.2^{cB}	1587.4 ± 417.1^{aB}

Each value is the mean ± standard deviation of four times. Identical superscript capital letters denote that are not significant difference among different cultivars of the same extracted solvent. Identical superscript small letters denote that are not significant difference among different extracted solvent of the same cultivar.

3.3. Antioxidant capacity determined by the ORAC assay using different colored rice strains

The antioxidant capacity of one white, two red, and two black rice cultivars was evaluated using the ORAC assay (Fig 5.1). The results showed that the antioxidant

capacity of different colored rice strains was in the same rank order as that obtained by the DPPH analysis method, but the values of antioxidant capacity obtained were different from those obtained with DPPH. For example, in Tsukushiakamochi, the value obtained by the ORAC assay (286.1μmol Trolox/g) was 70-fold that the DPPH assay (2065.8 μmol Trolox/100 mg) using the same solvent for extraction. Similar results were obtained for the other cultivars. Comparative analysis of the antioxidant capacity using DPPH and ORAC showed a difference with the method, and there was a rather lower correlation in tea and beverages according to Tabart et al. (2009). Our results partly agreeed with those of Tabart et al. in that the values showed a difference between DPPH and ORAC assays, but with a high correlation ($r = 0.98$) (Fig.5.2). It is possible to consider that the difference in correlation was caused by the difference in the kinds of phenolic compounds and in the ratio of different compounds. Moreover, the difference between the values obtained using DPPH and ORAC assays may result from the difference in reaction conditions; for example, DPPH is an electron reaction, whereas ORAC is a hydrogen atom transfer reaction. In brief, various methods of analysis should be examined so that the strongest antioxidant compound will be identified.

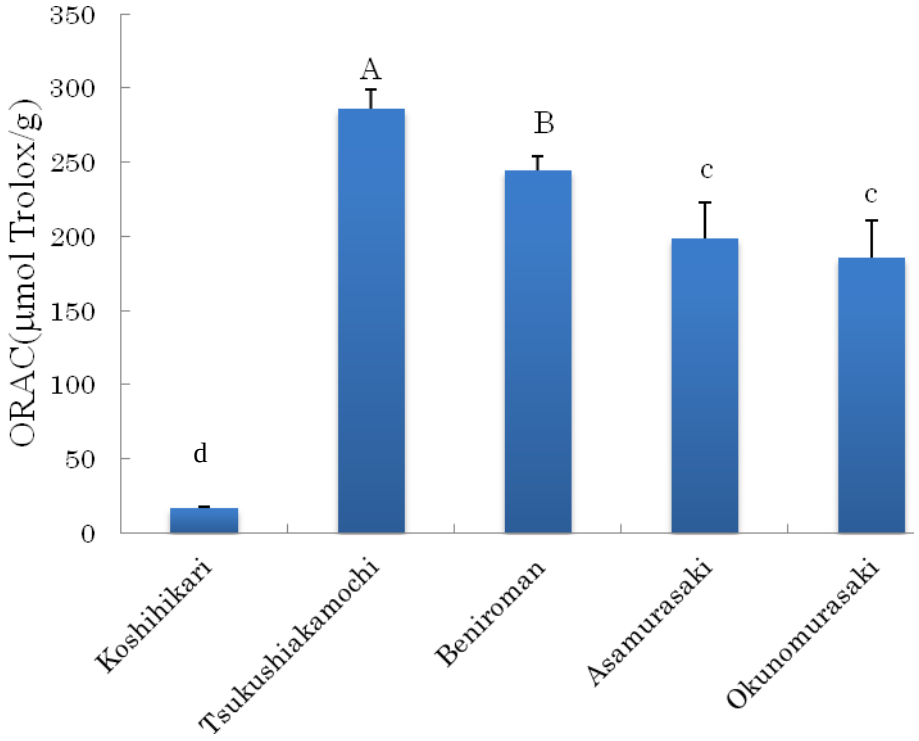


Fig.5.1 Evaluation of the antioxidant capacities of three different colored rice strains planted in 2007 year by ORAC assay.

Small letters denote that are significantly different at $p < 0.01$. Identical superscript letters A and B denote that are significantly different at $p < 0.05$.

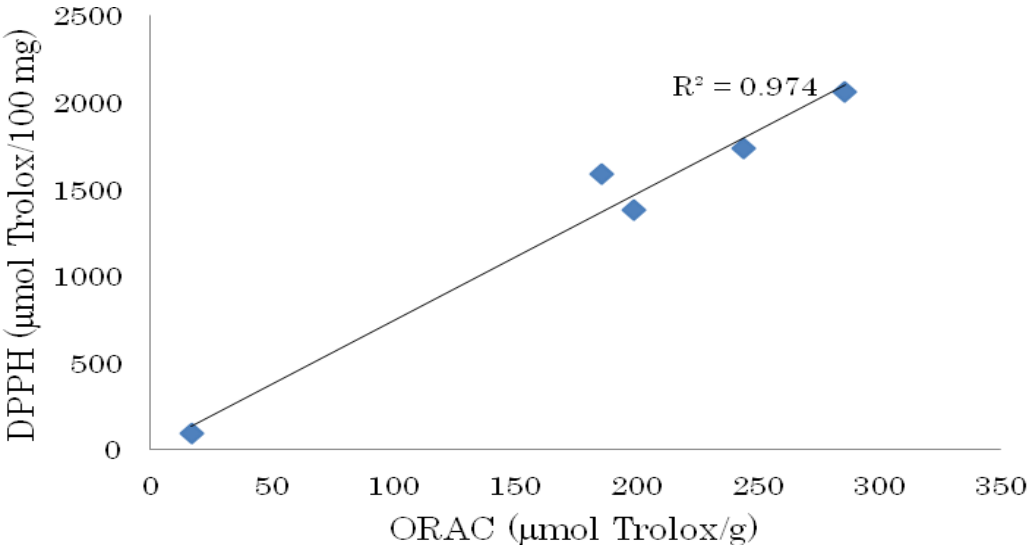


Fig 5.2 Correlation of four kinds of colored rice strains planted in 2007 between DPPH and ORAC

3.4. Antioxidant capacity in different planting years in different colored rice strains determined by DPPH assay

Table 5.4 Antioxidant capacity determined by DPPH method after extraction with 1% HCl methanol in five rice cultivars

Cultivars	DPPH (μmol Trolox/100 mg)		
	2005	2007	2011
White rice			
Koshihikari	85.8±21.8^{aC}	92.5±21.1^{aC}	95.67±12.2^{aC}
Red rice			
Tsukushiakamochi	1806.2±340.6^{aA}	2065.8±240.3^{aA}	2145.1±186.3^{aA}
Beniroman	1442.1±117.4^{bB}	1740.3±195.3^{aAB}	1722.6±52.7^{aA}
Black rice			
Asamurasaki	1411.3±10.4^{aB}	1383.8±239.8^{aB}	1703.4±118.4^{aA}
Okuromurasaki	1324.9±86.1^{aB}	1587.4±417.1^{aB}	1384.8±65.1^{aB}

Each value is the mean ± standard deviation of four times. Identical superscript capital letters denote that are not significant difference among different cultivars of the same planted year. Identical superscript small letters denote that are not significant difference among different planted year of the same cultivar.

The antioxidant capacity of one white, two red, and two black rice cultivars harvested in 2005, 2007 and 2011, were evaluated using the DPPH assay (Table 5.4). The results showed that there were no significant differences with the planting year except for Beniroman which showed a significant difference between 2005 and 2007 or 2011. For example, the value in Tsukushiakamochi planted in 2007 (2065.8 μmol Trolox/100 mg) was 259.6 μmol Trolox/100 mg higher than that in 2005 (1806.2 μmol Trolox/100 mg), and was 80μmol Trolox/100 mg lower than that in 2011 (2145.0 μmol Trolox/100 mg). Table 5.5 shows, the correlation between antioxidant capacity and rainfall, or temperature, or sunshine. The results showed a significant

positive correlation (r = 0.98) between sunshine and antioxidant capacity, but is significantly negative correlation (r = 0.92) between temperature and antioxidant capacity. However, rainful scarcely affected the antioxidant capacity. Therefore, we deduced that the antioxidant capacity is affected by the meteorological conditions, cultivation condition and genetic background.

Table 5.5 Relationship between meteorological condition and color hue or antioxidant capacity or total phenolic content (TPC) in Tsukushiakamochi

	Relationship (y = ax +b)[A]								
	Color value			Antioxidant capacity			TPC		
	a	b	r^B	a	b	r^B	a	b	r^B
Rainful of total	0.013	40.60	0.23	-4.09	2633.5	0.72	-2.76	810.26	0.66
Daily temperature	-2.47	98.21	0.78	-296.2	8684.9	0.92	-229.03	5550.5	0.96
Sun shine of total	0.03	38.36	0.35	8.32	804.43	0.98	6.10	-493.76	0.96

[A]: a and b are coefficients of the regression equation y = ax + b, [B]: correlation coefficients of the regression equation.

3.5. Comparison of total phenolics content (TPC) with different extraction methods in different colored rice strains

The phenolics contents in two black rice cultivars, two red rice cultivars and a white rice cultivar obtained with different extraction solvents are shown in Table 5.6. The results showed that there were significant differences in the TPC value with the extraction solvent, and the rank order was in the decreasing order of 1 % HCl methanol, 100% methanol, and 80% ethanol. For example, in red rice Tsukushiakamochi, the value obtained with 1% HCl methanol (416.5 mg/100 g) was higher than that obtained with either methanol (251.2 mg/ 100 g) or 80% ethanol

extract (186.4 mg/ 100 g). Similar to the results obtained in red rice cultivars, in black rice 1 % HCl methanol gave the highest TPC value; for example, the value in Okunomurasaki (403.2 mg/100 g) with 1% HCl methanol was about 4-fold higher than that obtained with 80% ethanol (101.3 mg/100 g) and that obtained with 100% methanol (128.9 mg/100 g). Zhao et al. (2006) reported that the rank order of TPC values in the same barley variety was in the decreasing order of 80% acetone, 80% ethanol, 80% methanol, and water. TPC value in flaxseed was the highest with 80% ethanol (3260 mg/100 g), followed by 100% methanol (2700 mg/100 g), and 80% methanol extract (2020 mg/100 g) (Anwar and Przybylski, 2012). Similarly, Sultana et al. (2007) reported that 80% aqueous ethanol was the most effective for extracting phenolic components from the bark of some plants. However, this study showed that the TPC values were in the order of 1% HCl methanol > 100% methanol > 80% ethanol extract. On the one hand, it is possible that different plants contain different phenolic components and different ratios. On the other hand, it is possible that different phenolic components can be selected depending on the extraction solvent. Moreover, the TPC value in white rice was 43.2, 12.7 and 11.3 mg/ 100 g, respectively, corresponding to the value with 1% HCl methanol, 100% methanol and 80% ethanol, and was all significantly lower than black and red rice cultivars. However, there was no significant difference between black and red rice strains as reported by Sompong et al. 2011 and Chen et al. 2012. In brief, a strong polar solvent gave a high TPC. It is reasonable to presume that phenolics dissolve easily in polar solvents.

Table 5.6 Phenolics content measured by UV spectrophotometry after extraction with different solvents in five rice cultivars planted in 2007

	TPC (mg /100 g)		
Cultivars	80% Ethanol	100% Methanol	1%HCl Methanol
White rice			
Koshihikari	11.3 ± 1.2^{bD}	12.7 ± 0.8^{bC}	43.2 ± 0.6^{aC}
Red rice			
Tsukushiakamochi	186.4 ± 13.1^{cB}	251.2 ± 4.1^{bA}	416.5 ± 3.6^{aB}
Beniroman	231.7 ± 5.2^{cA}	295.4 ± 11.3^{bA}	404.2 ± 1.0^{aB}
Black rice			
Asamurasaki	130.5 ± 3.4^{bC}	127.2 ± 17.9^{bB}	477.5 ± 0.8^{aA}
Okunomurasaki	101.3 ± 12.1^{bC}	128.9 ± 7.5^{bB}	403.2 ± 4.1^{aB}

Each value is the mean ± standard deviation of four times. Identical superscript capital letters denote that are not significant difference among different cultivars of the same extracted solvent. Identical superscript small letters denote that are not significant difference among different extracted solvent of the same cultivar.

3.6. Comparison of total phenolics content (TPC) for different year of planting in different colored rice strains

The phenolics contents with the year of planting after extraction with 1% HCl methanol in two black rice cultivars, two red rice cultivars and a white rice cultivar are presented in Table 5.7. The results showed significant differences with the year of planting in the same cultivar. For example, in red rice Tsukushiakamochi planted in 2005, 2007 and 2011, TPC values were 240.7, 416.5 and 500.6 mg/100 g, respectively; that in 2011 being 2-fold that in 2005. Similar to the results in red rice cultivars, in black rice Okunomurasaki the values were 305.4, 403.2 and 295.7 mg/100 g, respective in 2005, 2007 and 2011.

Table 5.7 Phenolics content obtained by extraction with 1% HCl methanol in five rice cultivars in different years of planting

	TPC (mg /100 g)		
Cultivars	2005	2007	2011
White rice			
Koshihikari	36.7 ± 1.2^{bD}	43.3 ± 0.6^{bC}	48.9 ± 0.7^{aD}
Red rice			
Tsukushiakamochi	240.7 ± 14^{cC}	416.5 ± 3.5^{bB}	500.6 ± 22^{aA}
Beniroman	337.2 ± 0.6^{bB}	404.2 ± 11.2^{aB}	389.8 ± 4.0^{aB}
Black rice			
Asamurasaki	509.1 ± 5.5^{aA}	477.5 ± 11.2^{bA}	534.6 ± 12.1^{aA}
Okunomurasaki	305.4 ± 11.7^{bB}	403.2 ± 4.1^{aB}	295.7 ± 1.7^{bC}

Each value is the mean ± standard deviation of four times. Identical superscript capital letters denote that are not significant difference among different cultivars of the same year planted. Identical superscript small letters denote that are not significant difference among different planted year of the same cultivar.

3.7. Correlation between TPC and meteorological conditions

There was a positive correlation (0.96) between TPC and sunshine hours (Table 5.6). When sunshine was under 160 hours, for example, in Tsukushiakamochi TPC was 240.7, 416.5 and 500.6 mg/ 100 g respective sunshine of 120.1, 155.2 and 157.6 hours. Similar to the results obtained in Tsushiakamochi, in Okunomurasaki with sunshine of 153.6 hours (403.2 mg/100 g) TPC was 97.8 mg/100 g higher than that with 108.7 hours (305.4 mg/100 g). However, TPC decreased slightly with sunshine time for more than 160 hours; for example, in Asamurasaki with sunshine of 185.5 hours (477.5 mg/100 g) TPC was 42.9 mg/100 g lower than that with 137.8 hours (534.6 mg/100 g), which was 25.5mg/100 g higher than that with 108.7 hours (509.1 mg/100 g). Furthermore, there was a significant negative correlation (0.96) between daily temperature and TPC. However, rainful scarcely affected TPC values. It is

reasonable to presume that sunshine contributes to phenolics synthesis, but a higher temperature affects the accumulation of phenolics.

3.8. Correlation between TPC and antioxidant capacity

Zhou et al. (2009) reported a high correlation between the content of total phenolic compounds and their antioxidant capacity in bayberry. In contrast, there was little correlation between TPC and antioxidant capacity in black and red rice strains (Chen et al., 2012). However, the results of this study were in accordance with those of Chen et al.; for example, TPC value in Tsukushiakamochi (416.5 mg/100 g) was 61mg/100 g lower than that in Asamursaki (477.5 mg/100 g), but the corresponding DPPH value was 682.0 μmol Trolox/100 mg higher in the same extract. On the other hand, there was a significant positive correlation in the same cultivar between TPC and antioxidant capacity; for example, Tsukushiakamochi planted in 2011 showed a higher TPC (500.6 mg/ 100 g) and a stronger antioxidant capacity (2145.1μmol Trolox/100 mg) than that planted in either 2005, 240.7 mg/100 g and 1806.2 μmol Trolox/100 mg, respectively, or 2007, 416.5 mg/100g and 1065.8μmol Trolox/100 mg, respectively. The results of the present study showed that the contents and ratios of antioxidant compounds differ with the plant and cultivar.

4. Conclusion

In brief, the present study revealed significant differences in the TPC and antioxidant capacity with the solvent mixture. However, the polar extraction solvent

might be the most suitable for analysis of antioxidant compounds and total phenolic content.

Chapter 6: Expressional analysis of flavonoid biosynthetic genes in different colored rice strains

1. Introduction

Colored rice contains rich flavonoids, and all flavonoids possess a basic benzopyrene skeleton, which is synthesized through the sequential condensation reaction of a ρ-coumarone-coenzyme A (ρ-coumaroyl-CoA) and three 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, methyl and sugar groups.

A number of studies have described the majority of genes and enzymes involved in the flavonoid biosynthetic pathway of maize, *Arabidopsis* and *Petunia* (Mol et al., 1998; Weisshaar and Jenkins, 1998; Holton and Cornish, 1995; Springob et al., 2003). 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) (Winkel-Shirley, 2001; Marles et al., 2003). The following produces a two-branched pathway, namely proanthocyanin and anthocyanin. Proanthocyanin is synthesized by leucoanthocyanidin reductase (LAR). In contrast, anthocyanin is synthesized by anthocyanidin synthase (ANS), and a portion of the anthocyanin is converted into proanthocyanin by anthocyanidin reductase (ANR).

Genes encoding homologs of CHS and CHI in rice were identified based on

sequence homology (Reddy et al., 1996; Druka et al., 2003). Similarly, a functional DFR gene was revealed when over-expressing transgenic rice was found to produce red-colored pericarp (Furukawa et al., 2007). ANS, a key enzyme in the conversion of colorless leucoanthocyanidin to colored anthocyanidin, was initially isolated from maize, snapdragon, and *Petunia* (Holton and Cornish, 1995). ANS was recently detected in rice by homology research (Reddy et al., 2007). However, it remains unclear whether upstream regions of the flavonoid biosynthetic pathway affect downstream regions gene expression in monocot rice. Additionally, no report has shown differences in the regulation of gene expression of the flavonoid biosynthetic pathway between different colored rice strains, or in different tissues of the same colored rice strain. Moreover, the relation between anthocyanin pigment accumulation and the flavonoid biosynthetic pathway, as a whole, has not been reported in rice.

This study describes the expression of five genes involved in the flavonoid biosynthesis pathway, from four different colored rice strains, in various developmental stages and tissues.

2. Materials and methods

2.1. Plant material and growth conditions

The following *japonica* rice (*Oryza sativa* L.) lines were used for expression analysis of flavonoid biosynthetic genes in different colored rice strains: white rice Nipponbare (improved, Japan), black rice Chinakuromai (semi-improved, China), red

rice Tsukushiakamochi (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 October, 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. Tillering leaf RNA was extracted at the same time as for sheath RNA. Flag leaf RNA was extracted 7 days after flowering.

2.2. RNA extraction

Total RNA was extracted from roots, leaf, sheath, seed capsule and endosperm of colored rice strains that were black rice (Chinakuromai), red rice (Tsukushiakamochi), green rice (Akunemochi), and white rice (Nipponbare) cultivars. RNA was extracted using a Trizol (Invitrogen) RNA mini-kit following the manufacturer's protocol (Qiagen, Valencia, CA, USA). Extraction methods were as follows: A. Frozen samples were homogenized with a mortar and pestle in liquid N_2. The ground powder was transferred to empty falcon tubes on in liquid N_2 until the homogenization procedure was ready to be performed. B. 1 ml of Triz buffer was added, and was shaken with a vortex for only 10 s. Then incubate the lysate with TRIZOL Reagent at room temperature for 5 min to allow complete dissociation of nucleoprotein complexes. C. Add 0.2 ml chloroform or 50 µl 4-Bromoanisole per 1 ml TRIZOL Reagent used. Shake the tube vigorously by hand for 15 s. D. Incubate at room temperature for 3 min. E. Centrifuge the sample at 15000rpm for 15 min at 4 °C. F.

Transfer 600 µl of the colorless, upper phase containing the RNA to a fresh RNase-free tube. G. Add an equal volume of 70 % ethanol to obtain a final ethanol concentration of 35 %. Mix well by vortexing. H. Washing and elution. I. The quantity of total RNA was determined by measuring absorbance at 260 nm and 280 nm by using a spectometre. In addation, the level of protein contamination in the RNA was determined based on the A260/280 ration. Only RNA samples with rations of 2.0-2.2 were used for these experiments.

2.3. cDNA transcription

cDNA was synthesized from 1 µg of total RNA using the iScript cDNA Synthesis kit (Bio-Rad). The transcription method are as followes: A. Reverse transcription using either anchored-oligo (dT)18 primer. B. Reaction system: total RNA 1 µg, primer 2.5 µm, PCR-grade water to 11.4 µl volume and denature the template-primer mixture by heating the tube for 10 min at 65 °C in a block cyaler with a heated lid. C. Then, transcriptor high fidelity reverse transcriptase reaction buffer 4 µl, protector RNase inhibitor 0.5 µl, deoxynucleotide mix 2 µl, DTT 1 µl, transcriptor high fidelity reverse transcriptase 1.1 µl were added, and total volume come to 20 µl. D. Incubate the reaction for 30 min at 55 °C. E. Then, inactive transcriptor high fidelity reverse transcriptase by heating to 85 °C for 5 min.

2.4. Real-time Reverse Transcription-qPCR Analysis

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 (Finnzymes, Agilent Technologies, CA, USA), according to the manufacturer's instructions. Reactions were performed in triplicate using 10 μl of 2 × SYBR Green QPCR master mix, 1 μM of each primer (Supplement in Table 6.1), 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: tgagacaccatcaccggaatc; reverse primer: atccaaaggccaatcgtgaa) to minimize variation in cDNA template levels. Relative expression levels were calculated using the comparative threshold (Ct, cycle value) method (Livak and Schmittgen, 2001). Mean values were obtained from three biological replicates, each determined in triplicate.

Table 6.1 List of RT-PCR primers of flavonoid genes

Locus ID		Primer	Sequence (5' → 3')	Product size/bp
CHS	AC136226	F	catggactatttggggatgg	226
		R	aatgtgtcgagcaagcactg	
CHI	XM-470129	F	agaagttcacgagggtgacg	195
		R	gagtgggtgaagaggatgga	
DFR	Y07956	F	gtcaagatgaccggatggat	217
		R	aactgcacctgcttcaggat	
ANS	Y07955	F	ctctcctgggtcgtcttctg	184
		R	tatcattccgttcgatgcag	
LAR	BN000704	F	cttcatctgctgcaactcca	195
		R	gtgcacgatcttgttgatgc	

3. Results and discussion

3.1. 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 Fig.6.1. The transcript levels of flavonoid biosynthesis gene varied according to the gene, tissue, and colored rice strain. The transcript level of the CHS gene, which catalyzes the first step of the phenylpropanoid pathway leading to flavonoid production, varied greatly depending on tissue type and colored rice strain (Fig. 6.1 CHS). The transcript level in the root of red rice was the highest

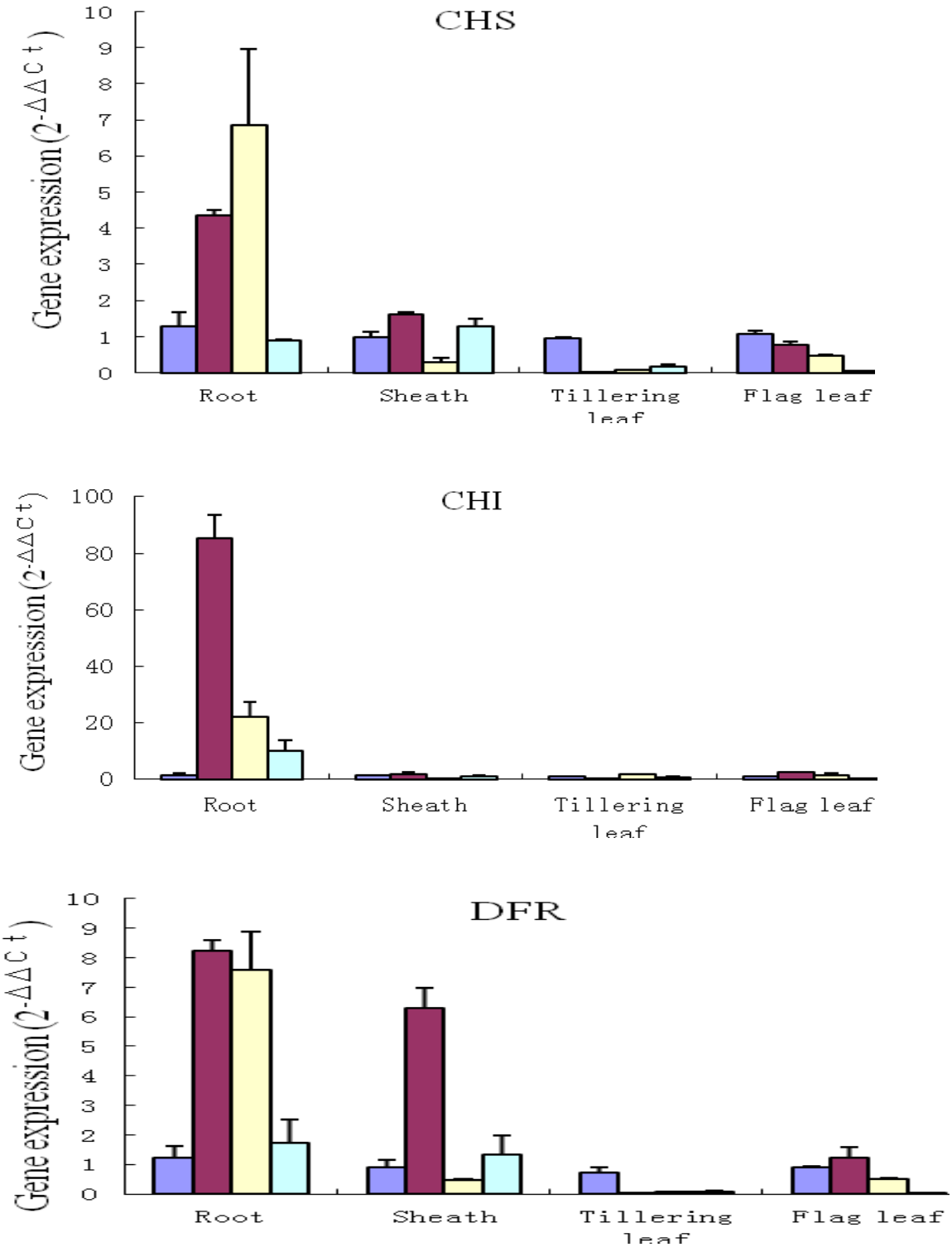


Fig. 6. 1 Transcript levels of flavonoid biosynthesis-related genes in different tissues of four colored rice strains

White rice Green rice Red rice Black rice

Error bars represent SD values for the means of triplicate.

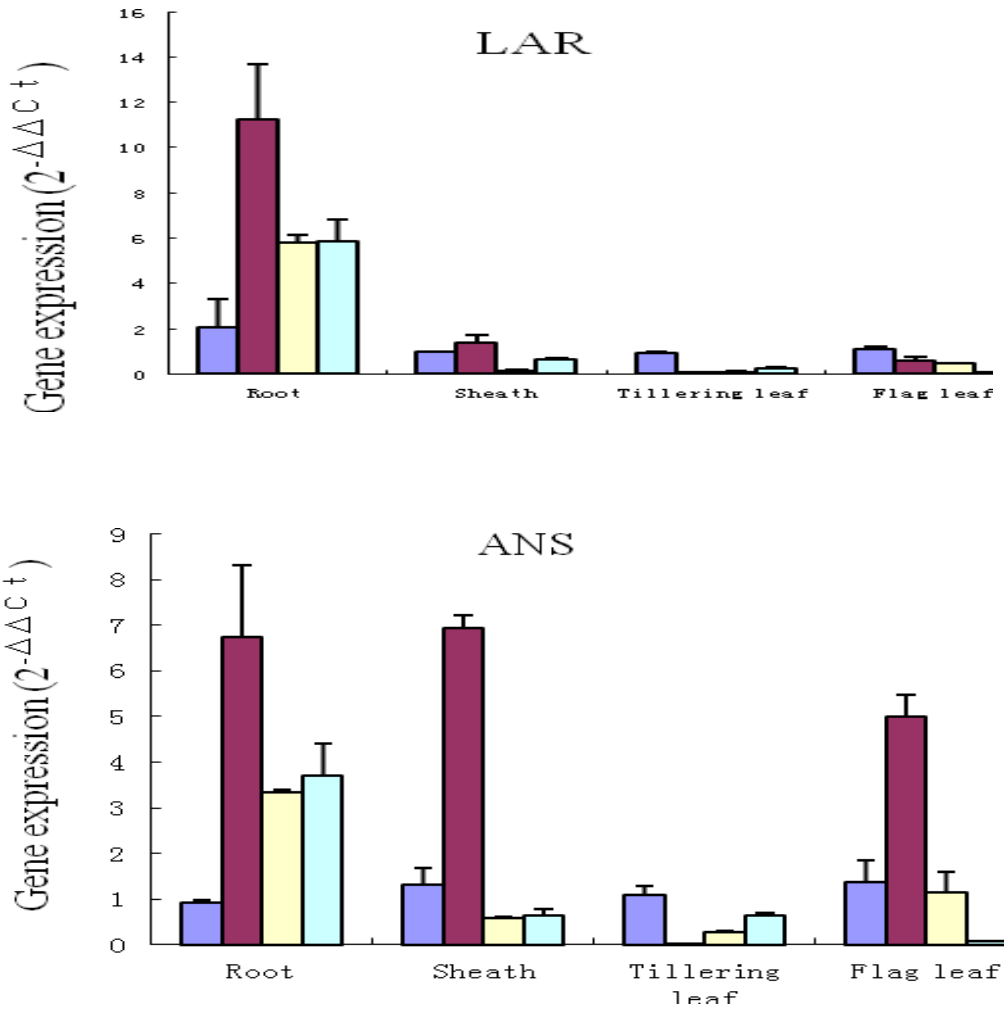


Fig. 6.1 Continued

among the different colored rice strains. Moreover, significant differences were observed in the different tissues of the same colored rice; CHS transcript levels in the root of red rice was almost 20-fold higher than those of sheath and flag leaf, while CHS transcript was barely detected in tillering leaf. Likewise, the root of green rice presented higher transcript levels than other tissues, such as sheath and flag leaf, but expression was almost completely absent in tillering leaf. The transcript levels were lower in all tissues of black rice compared to other rice strains, and was barely detected in flag leaf.

DFR was present at significantly higher transcript levels in the root and sheath of

green rice, and the root of red rice (Fig. 6.1 DFR). In contrast, expression was almost absent in tillering leaf of green rice (0.0009). Levels increased gradually with plant maturation in flag leaves (1.23). Additionally, significant differences were observed in the roots of different colored rice strains; for example, green rice showed a 7-fold greater transcript level than red rice.

ANS was presented in all tissues of all colored rice strains, including common (white) rice (Fig. 6.1 ANS). Results similar to CHS, CHI and DFR were observed, where transcript levels varied according to tissue; for example, root transcript level was 46-fold higher than in flag leaf, and≥ 5 -fold higher than that of sheath and tillering leaf.

High LAR transcript levels were presented in the root of all colored rice strains (Fig.6.1 LAR), with the following rank order: green (11.27) > black (5.87) > red (5.82) > white rice (2.04). However, transcript levels were lower in other tissues, for example, 0.96, 1.36, 0.16 and 0.64 in the sheath of the green, black, red and white rices, respectively.

The transcript levels of CHS, CHI, and DFR showed a similar tendency. Higher transcript levels were observed in the root, particularly low levels in tillering leaves, which subsequently increased in later development. In addition, fluctuations in the expression of the five flavonoid biosynthetic genes indicated that transcript levels of the up-stream genes did not coordinate with those down-stream genes of flavonoid biosynthetic pathway (Fig.6.2). For instance, the transcript level of CHS in the root of

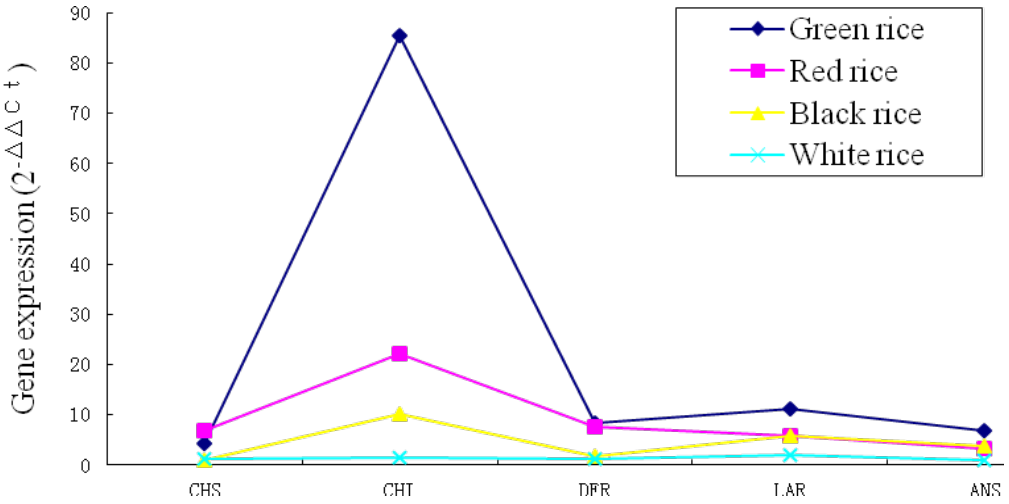


Fig. 6.2 Transcript levels of flavonoid biosynthesis-related genes in the roots of four colored rice strains

red rice (6.85) was higher than that of green rice (4.36); whereas, CHI transcript level of red rice was only 22.15, which is much lower when compared to that of green rice (85.39). However, minimal differences in expression among the down-stream genes of flavonoid biosynthetic pathway were observed. Therefore, it is possible that the genes are regulated independently of each other. Furthermore, differences in transcription were observed, for example, there were much higher transcript levels observed in green rice compared to black rice.

3.2. Analysis of transcript levels of flavonoid gene expression during grain filling

Caryopsis RNA was extracted at 3, 5, and 7 days after flowering. Endosperm RNA was extracted at 5 days after flowering. The results are presented in Table 6. 2, which shows that white rice transcript flavonoid biosynthesis genes throughout caryopsis development and in the endosperm, and there were not almost varied in different

checked stage and tissue. Furthermore, transcript levels were lower than in colored rice. In contrast, there were significant and similar variations in different stages of green, red and black rice, where caryopsis transcript levels reached dramatically high levels in second stage (5 days), with a subsequent decrease in the third stage (7 days). The results also showed that the various flavonoid biosynthetic genes were present at different transcript levels in different colored rice strains and at different developmental stages, as seen in Fig.6.1. For example, DFR in the first and second stages of red rice showed 2000-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 green and red rice, respectively. LAR transcript levels in red rice were the highest among the colored rice. LAR transcript levels at the second stage in different colored rice were in the rank order: red rice (751.78) > black rice (100.08) > green rice (32.56) > white rice (0.98). Additionally, all gene transcript levels in the different development stages were significantly increased or decreased, from 2-fold to hundreds-fold, in black rice, while CHI in the second stage was about 43- and 12-fold greater than the first and third stages, respectively. Likewise, ANS in the second stage was about 315- and 18-fold greater than the first and third stages, respectively. Furthermore, this data shows that the transcript of ANS and LAR, which are derived from the common genes up-regulated

Table 6.2 Transcript levels of flavonoid biosynthetic genes in caryopsis and endosperm

	Gene expression ($2^{-\Delta\Delta Ct}$) of caryopsis[a]			Gene expression($2^{-\Delta\Delta Ct}$) of endosperm[a]
	3 days	5 days	7 days	
White rice				
CHS	1.08±0.09	0.92±0.14	0.92±0.14	0.98±0.22
CHI	1.01±0.10	1.08±0.07	1.10±0.03	1.56±0.23
DFR	2.05±0.91	0.63±0.30	0.85±0.03	1.37±0.09
ANS	1.06±0.09	1.42±0.33	1.15±0.17	1.25±0.25
LAR	1.32±0.35	0.98±0.03	1.07±0.03	1.27±0.19
Green rice				
CHS	0.29±0.05	3.85±1.02	2.42±0.93	3.65±0.88
CHI	2.06±0.09	14.90±6.16	1.57±0.24	17.66±7.79
DFR	5.48±2.57	32.56±0.85	1.18±0.13	24.91±2.98
ANS	0.49±0.11	113.37±35.0	4 2.57±0.69	2.23±1.14
LAR	0.30±0.13	32.56±0.85	1.32±0.30	1.77±0.77
Red rice				
CHS	0.23±0.03	8.15±1.40	3.24±0.19	nd
CHI	3.01±1.10	54.26±14.45	1.12±0.08	nd
DFR	2666.63±401.25	5350.60±726.74	23.25±1.05	0.06±0.003
ANS	1.67±0.14	73.35±22.59	5.18±1.89	nd
LAR	42.51±0.63	751.78±71.08	4.80±1.71	nd
Black rice				
CHS	2.00±0.39	71.86±8.18	3.13±0.32	5.89±0.66
CHI	0.69±0.13	29.66±6.23	2.51±0.60	2.27±1.61
DFR	2.62±0.13	798.66±115.07	191.0±78.03	9.39±3.70
ANS	1.89±0.58	595.30±24.08	32.43±1.70	2.87±0.32
LAR	1.27±0.11	100.08±15.70	1.58±0.48	3.17±0.17

[a] Values expressed as means ±SD (n = 3). RNA of caryopsis were extracted from 3, 5 and 7 days after flowering. RNA of endosperm were extracted from 5 days after flowering. Transcript levels were quantified by reverse transcription (RT)-qPCR (for details, see materials and methods).

by DFR in the flavonoid biosynthetic pathway, is correlated; for example, Table 5.2 shows that during conditions resulting in elevated expression of DFR, LAR (751.78) transcription was 10-fold higher than that of ANS (73.35) in red rice. In contrast, the ANS (595.30) transcript level was about 6-fold higher than that of LAR (100.08) in

black rice.

Flavonoid biosynthesis gene transcript levels varied in the endosperm of different colored rice strains (Table 6.2). In brief, levels in black rice and green rice was higher than in white and red rice, and levels were barely detected in red rice.

3.3. Discussion

Low transcript levels for the five flavonoid biosynthesis genes were observed in all white rice tissues, and these levels showed minimal variation, especially during caryopsis formation (Fig.6.1 and Table 6.2). In contrast, significant differences in gene expression were observed among the three colored rice strains, and occurred to specificity for spatial and temporal. The DFR gene was detected in over-expressing transgenic rice observed to produce red-colored pericarp (Furukawa et al., 2007). Table 6.2 shows that red rice caryopsis possessed high DFR transcript levels (5350.60). According to Furukawa's result, it is reasonable to assume that the red pericarp is derived mainly from the strong expression of DFR. Additionally, Table 6.2 shows that red rice contained a rather high LAR (751.78) transcript level. In contrast, black rice showed a relatively high ANS transcript level compared to LAR. This might provide a reasonable explanation for the presence of visible anthocyanin pigments in black rice, while red rice is rich in proanthocyanin (Chen et al., 2012). Low transcript levels of flavonoid biosynthesis genes were detected in white rice, while high levels were found in red and black rice. It is reasonable to assume that the presence of a strong promoter or other factors that induce the up-stream gene

transcription of the flavonoid biosynthetic pathway, leading to a dramatic accumulation of anthocyanin and proanthocyanin in black and red rice. For example, *long hypocotyl* 5 (a Leu-zipper TF) and *phytochrome-interacting transcription factor* 3 (PIF3) bind directly to the promoters for anthocyanin structural genes, such as CHS, CHI, F3H, DFR and LDOX, in *Arabidopsis* (Lee et al., 2007; Shin et al., 2007). Further research is required to elucidate the triggers responsible for the dramatic increase in flavonoid biosynthetic gene transcription. Reddy et al. (2007) reported that increased accumulation of anthocyanin was accompanied by a concomitant decrease in proanthocyanidin. Consistent with the result of Reddy et al., there exists a strongly competitive mechanism between anthocyanin and proanthocyanidin, as shown in Table 6.2. This could perhaps explain the lack of color in common rice, the high anthocyanin content of black rice, and the rich proanthocyanin content of red rice.

The transcription of anthocyanin biosynthetic genes appears to be highly coordinated in grape skin during berry development and in bilberry fruit development (Boss et al., 1996； Jaakola et al., 2002). 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 observed to be controlled by the formation of metabolons (Burbulis and Winkel-Shirley, 1999; Pelletier, 1999). However, Clive-Lo and Nicholoson (1998) reported that the expression of flavonoid biosynthesis genes was not coordinated in sorghum mesocotyls. Similar to the results of Clive-Lo and Nicholson, this study indicated that

the five flavonoid biosynthesis genes analyzed were independently expressed in monocot rice (Fig.5. 2). We assume that differences exist between monocot and dicot expression modules, at least with respect to rice.

4. Conclusion

Expression of flavonoid biosynthesis genes existed in all colored rice strains including white rice, and occurred to specificity for spatial and temporal among red black and black rice strains. Besides, this study provides a good elucidation for expression difference between red and black rice, and cause of colorless endsoperm and so on. It may be provide a proof for breeding of colored rice cultivars.

Chapter 7: Sugar inducing flavonoid biosynthetic genes response

1. Introduction

Sugar is a common regulator of gene expression for metabolic enzymes and proteins involved in photosynthesis, carbohydrate metabolism, pathogenesis (Rolland et al., 2006), and anthocyanin biosynthesis (Baier et al., 2004). In addition, Baier et al. (2004) reported that the interrelationships between developmental, environmental, and metabolic signal transcription 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 (Tsukaya et al., 1991), while *Petunia* corollas cultured *in vitro* without Suc were devoid of pigmentation (Weiss, 2000). DFR and ANS were strongly increased by Suc in grape (*Vitis vinifera*) cells (Gollop et al., 2001; 2002). 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 (Lloyd and Zakhleniuk, 2004). A similar phenomenon occurred in response to glucose, which induces flavonoid biosynthesis in *Arabidopsis* (Kwon et al., 2011; Solfaneli et al, 2006); however, responses to Suc and Glc in rice require clarification.

This study describes the expression of five genes involved in the flavonoid

biosynthetic pathway by sugar treatment.

2. Material and method

2.1. Plant material and growth conditions

That black rice (Chinakunomai) was used for reaction induced, was grown in potting soil in a green house for 30 days. Then plant was treatment by sugar.

2.2. Sugar treatments

Black rice was grown in potting soil in a green house for 30 days. Next, they 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. RNA were extracted from 30 h after treatment with the sugar solutions.

2.3. RNA extraction

Extract method was described as Chapter 6.

2.4. cDNA transcript

Transcript method was described as Chapter 6.

2.5. Real-time Reverse Transcription-qPCR Analysis

The method was described as Chapter 6.

3. Result and discussion

3.1. Results

To identify how genes involved in anthocyanin biosynthesis in rice are regulated by sugar, we analyzed flavonoid biosynthetic pathway genes in the absence (control) or presence of exogenous Suc or Glc, and performed a real-time PCR experiment with seedlings treated with Suc (0.2, 0.4, 0.6 M), Glc (0.2, 0.6 M), and mixture of Suc 0.2 M and Glc 0.2 M, respectively. The results showed in Fig.7.1 demonstrate that the transcript levels of DFR, LAR, and ANS were strongly and dose-dependently induced in response to Suc; for example, 0.2 M Suc strongly induced DFR and ANS transcript

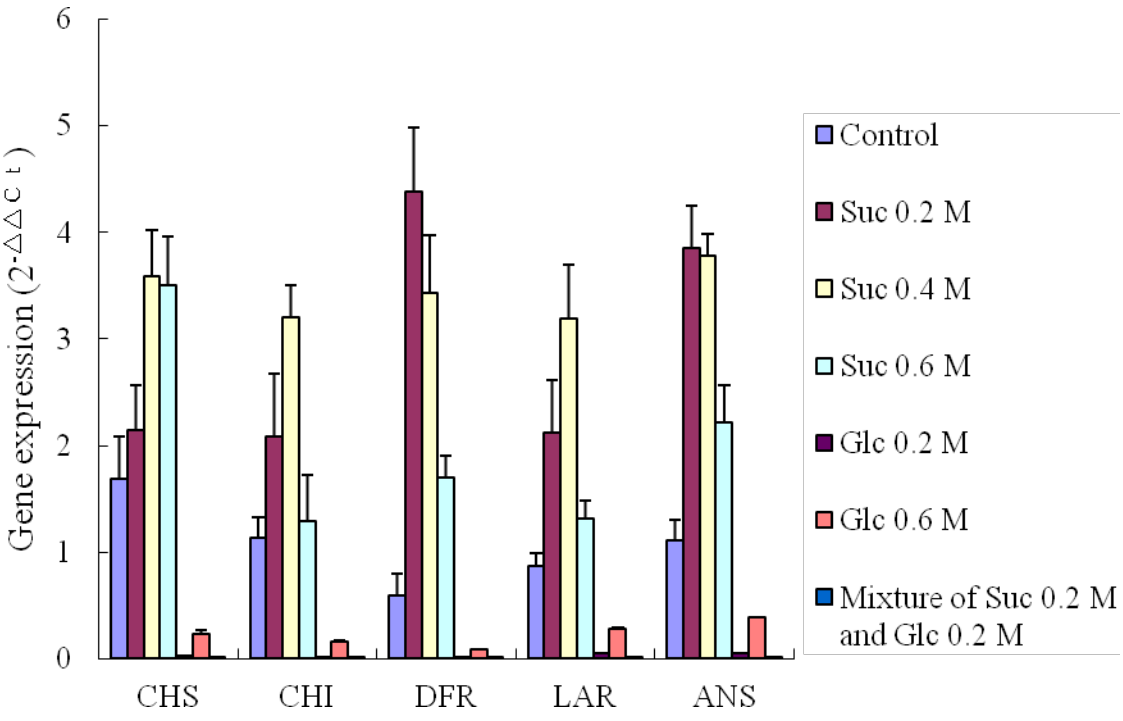


Fig.7.1 The effect of sugar treatment on flavonoid biosynthesis-related gene transcript levels

Error bars represent SD values for the means of triplicate.

levels to about 7- and 4-fold greater than control, respectively, with a subsequent decrease with increasing concentration. Treatment with 0.4 M Suc resulted in the highest LAR, CHI and CHS transcript levels. In contrast, Glc 0.2 M, 0.6 M, and a mixture of 0.2 M Glc and 0.2 M Suc repressed flavonoid biosynthetic gene transcript. For example, 0.2 M Glc strongly repressed DFR transcript levels to 50-fold lower than control. Likewise, treatment with mixture of Glc and Suc for DFR transcript level was 200-fold lower than that of control. ANS, CHI, LAR and CHS presented similar DFR results. In brief, in this study, Glc negatively regulated flavonoid biosynthesis gene transcription.

3.2. Discussion

A number of studies have investigated the sugar-induced accumulation of anthocyanin; for example, genes encoding for DFR and ANS were up-regulated and anthocyanin accumulation was significantly increased by exposure to Suc in grape cells (Gollop et al., 2002). Solfanielli et al. (2006) reported that flavonoid and anthocyanin biosynthesis pathway genes were strongly up-regulated following Suc treatment, affecting both flavonoid and anthocyanin contents in *Arabidopsis*. This was particularly evident for CHS, CHI and DFR. Excised *Arabidopsis* leaves fed Suc showed anthocyanin accumulation and increased levels of CHS, CHI and DFR mRNAs (Tsukaya et al., 1991; Mita et al., 1997). The expression of grape DFR is responsive to Suc (Gollop et al., 2002). Suc, but not Glc and Fru, induced PAP1 and ANS mRNA levels in *Arabidopsis* and triggered a dominant increase in anthocyanin

synthesis (Solfanelli et al., 2006). In contrast, Gollop et al. (2001; 2002) 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, only Suc significantly induced the transcription of CHS, CHI, DFR, ANS and LAR. Our results showed the negative regulation of flavonoid biosynthetic genes with 0.2 M and 0.6 M Glc and a mixture of Suc and Glc (Fig.7.1). It is reasonable to assume that the underlying mechanisms might be distinct from the sugar-specific mechanisms active in different plants, for example, non-specific sugar responses in grape, whereas all induce anthocyanin biosynthesis and increase DFR expression. However, they are greatly sensitive in *Arabidopsis* and rice, and only Suc was able to strongly induce accumulation of the transcription of flavonoid biosynthetic genes.

Solfanelli et al. (2006) reported that down-stream 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 transcript 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. For example, Jeong et al. (2010) reported that ethylene inhibited anthocyanin accumulation in *Arabidopsis* seedlings grown in the presence of Suc and light. The *Arabidopsis pho* 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 (Lloyd and Zakhleniuk, 2004). Expression of the flavonoid biosynthesis pathway transcription factors *PAP1* and *PAP 2* result in enhanced CHS and DFR expression in *Arabidopsis* (Borevitz et al., 2000). In *Arabidopsis*, DFR expression is under the control of TT2, which interacts with TT8 (Nesi et al., 2001). Therefore, there is continued interest in research into how up-stream factors stimulate or suppress the accumulation of anthocyanin in response to Suc and Glc in rice.

4. Conclusion

According to induced study using Glc and Suc in rice, the results showed that the transcription of DFR, LAR, CHI, CHS and ANS were strongly and dose-dependently induced in response to Suc, and different gene occurred in difference of reaction induced,

Chapter 8: White light inducing flavonoid biosynthetic genes response

1. Introduction

Light is an important environmental factor regulating plant development and gene transcription. Anthocyanin pigment accumulation and CHS transcript levels were increased in response to light in the seedlings of species such as *Arabidopsis* (John and Gareth, 1996). To date, there has been considerable research regarding blue and UV light, indicating that specific photoreceptors are largely responsible for the gene activation and pigment accumulation induced by visible wavelengths (Feinbaum et al., 1991; Kubasek et al., 1992; Batschauer et al., 1996; Bosl et al., 1998); however, little research in rice has been conducted with respect to white light (Jiao et al., 2005). Moreover, no reports exist that deal with flavonoid biosynthesis gene expression in response to white light.

This study describes the transcription of five genes involved in the flavonoid biosynthetic pathway by white light treatment.

2. Material and method

2.1. Plant material and growth conditions

That black rice (Chinakuromai) was used for reaction induced, was grown in potting soil in a green house for 30 days; the plants were used for light treatment.

2.2. Light treatments

Black rice was grown in potting soil in a green house for 30 days. For light treatments, plants were transferred to a controlled environment growth cabinet, arranged randomly within the cabinet and spaced to prevent shading. Plants were grown under either 12, 16 or 24 h photoperiod (6660 lux), at a constant 25 °C and 65% humidity for a period of 3 days. 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.

2.3. RNA extraction

Extract method was described as Chapter 6.

2.4. cDNA transcript

Transcript method was described as Chapter 6.

2.5. Real-time Reverse Transcription-qPCR Analysis

The method was described as Chapter 6.

3. Results and discussion

3.1. Results

To investigate the anthocyanin biosynthesis genes in rice that are regulated by

white light, we performed real-time PCR using RNA from seedlings treated with 12, 16, or 24 h of white light. Fig.8.1 shows that white light induced the transcription of all flavonoid biosynthetic genes, with exposure-dependent differences observed. DFR, LAR, ANS and CHI mRNA 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 transcription, increasing 8- and 9-fold at 16 and 24 h, respectively. Likewise, maximal CHS transcript level was observed at 16 h (7-fold greater than 12 h). Subsequently, levels decreased with increasing exposure time; CHS at 24 h was only 5-fold higher than that of 12 h.

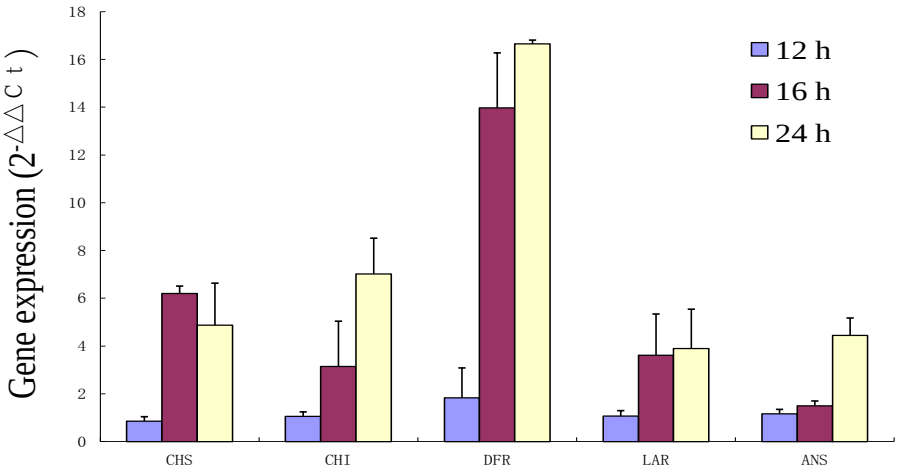


Fig.8.1 The effect of white light treatment (12, 16 and 24 h) on biosynthesis-related gene transcript levels

Error bars represent SD values for the means of triplicate.

3.2. Discussion

In low fluence UV-B responses, a very short period of exposure could induce anthocyanin biosynthesis in rice (Reddy et al., 1996) and maize (Singh et al., 1999). Our research showed that white light could induce transcription of anthocyanin biosynthesis gene, but it is not stronger than UV-B responses. It is reasonable to assume that transcription level of ANS to degree of sensitivity of different light source exist in difference in rice. In parsley cells, UV radiation induced a transient increase in the transcription rate of CHS (Schulze-Lefert et al., 1989). Moscovici et al. (1996) reported that light was essential for ANS and CHS expression in the detached corolla limbs 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 (Zhou et al., 2007). F3H, DFR and ANS gene expression in sorghum mesocotyl dramatically decreased with light treatment, whereas that for CHS significantly increased (Clive-Lo and Nicholoson, 1998). It was observed that in *Arabidopsis* cell culture grown under low fluence white light resulted in very low CHS transcript levels induced in mature leaf tissue (Feinbaum and Ausubel, 1988; Jackson et al., 1995). The flavonoid biosynthetic genes 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 (Albert et al., 2009). Our study showed that white light could induce the transcription of genes of the entire flavonoid biosynthetic pathway; however, differences were observed in the degree of sensitivity and the required

illumination time (Fig.8.1). Moreover, the flavonoid biosynthetic mechanism response in different plants differs according to the illumination source, at least for grape, *Arabidopsis* and rice. Concurrently, differences were found in the light-induced expression of up- and down-stream genes in the flavonoid biosynthetic pathway between *Arabidopsis* and rice.

4. Conclusion

White light can induce the transcription of all flavonoid biosynthetic genes, and transcript levels significantly increased depending on the length of exposure to white light.

Conclusion

Colored rice cultivars, including green rice, showed stronger antioxidant and free-radical scavenging activities than the white rice cultivar. Furthermore, TAC was related to the color of rice; namely, the deeper the color, the higher TAC. In contrast, significant differences in TPC were observed within rather than between colored cultivars. Genes expression of flavonoid biosynthetic pathway existed in specificity for spatial and temporal in colored rice strains, but not in white rice. Lastly, light and sugar all could affect genes expression of flavonoid biosynthetic pathway. Light induced positively genes expression of flavonoid biosynthetic pathway. Likewise, Suc strongly induced genes expression, but Glc strongly suppressed genes expression. Therefore, Suc is specific for rice. Besides, this paper will be useful for breeding, food processing, agronomy and plant physiology field in the near future.

References

Abdel-Aal E.S.M., & Hucl P. (1999). A rapid method from quantifying total anthocyanins in blue aleurone and purple perciarp wheats. *Ceral. Chem.*, 76: 350-354.

Abdel-Aal E.S. M., Young J. C. & Rabalski I. (2006). Anthocyanin composition in black, blue, pink, purple, and red cereal grains. *J. Agric. Food Chem.*, 54: 4696-4704.

Acquaviva R., Russo A., Galvano F., Galvano G., Barcellonam L., Li Volti G., & Vanella A. (2003). Cyanidin and cyaniding. 3-O-beta-D-glucoside as DNA cleavage protectors and antioxidants. *Cell Biol. Toxicol.*, 19: 243-252.

Albert N.W., Lewis D.H., Zhang H.B., Irving L.J., Jameson P.E., & Davies K.M. (2009). Light-induced vegetative anthocyanin pigmentation in Petunia. *J. Ext. Bot.*, 60: 2191-2202.

Anar F., & Przybylski R. (2012). Effect of solvents extraction on total phenolics and antioxidant activity of extracts from flaxseed (*Linum Usitatissimum* L.). *Acta Sci. Pol., Technol. Aliment*, 11: 293-301.

Antolovich M., Prenzler P., Robards K., & Ryan D. (2000). Sample preparation in the determination of phenolic compounds in fruits. *Analyst*, 125: 989-1009.

Baier M., Hemman G., Holman R., Corke F., Card R, Smith C., Rook F., & Bevan M.W. (2004). Characterization of mutants in Arabidopsis showing increased sugar-specific gene expression, growth, and developmental response. *Plant*

Physiol., 134: 81-91.

Batschauer A., Rocholl M., Kaiser T., Nagatani A., Furuya M., & Schafer E. (1996). Blue and UV-A light-regulated CHS expression in Arabidopsis independent of phytochrome A and phytochrome B. *Plant J.*, 9: 63-69.

Bhattacharjee P., Singhal R.S., & Kulkarni P.R. (2002). Basmati rice: A review. *Int. J. Food Sci. Tech.*, 37: 1-12.

Boase M.R., Davies K.M. (2006) Modification of flower colour and plant form in selected ornamentals by molecular breeding**.** Global Science Books, 504-511.

Bonoli M., Verardo V., Marconi E., & Caboni M.F.(2004). Antioxidant phenols in barley (*Hordeum vulgare* L.) flour: comparative spectrophotometric study among extraction methods of free and bound phenolic acids. *J. Agric. Food Chem.*, 52: 5195-5200.

Bonoli M., Marconi E., caboni M.F. (2004). Free and bound phenolic compounds in barley (*Hordeum vulgare* L.) flour evaluation of extraction capability of different solvent mixtures and pressurized liquid methods by micelllar electrokinetic chromatography and spectrophotometry. *J. Chromatogr. A.*, 1057: 1-12.

Borevitz J.O., Xia Y., Blount J., Dixon R.A., & Lamb C. (2000). Activation tagging identifies a conserved MYB regulator of Phenylpropanoid biosynthesis. *Plant Cell*, 12: 2383-2394.

Brenda W.S. (2001). Plant it takes a garden how work on diverse plant species has contributed to an understanding of flavonoid metabolism. *Physiol.*, 127: 1399-1404.

Brown C. R., Culley D., Yang C. P., Durst R., & Wrolstad R. (2005). Variation of anthocyanin and carotenoid contents and associated antioxidant values in potato breeding lines. *J. Am. Soc. Hortic. Sci.*, 130: 174-180.

Burbulis I.E., & Winkel-Shirley B. (1999). Interactions among enzymes of the Arabidopsis flavonoid biosynthetic pathway. *Proc. Natl. Acad. Sci.* USA, 96: 12929-12934.

Chatha S.A.S., Anwar F., Manzoor M., & Bajwa J.R..(2006). Evaluation of the antioxidant activity of rice bran extracts using different antioxidant assays. *Grasas Aceites*, 57: 328-335.

Chen X.Q., Nagao N., Itani T., & Irifune K. (2012). Anti-oxidative analysis, and identification and quantification of anthocyanin pigments in different colored rice. *Food Chem.*, 135: 2783-2788.

Choi S.P., Kang M.Y., Koh H.J., Nam S.H., & Friedman M. (2007). Antiallergic activities of pigmented rice bran extracts in cell assays. *J. Food Sci.*, 72: 719-726.

Clive-Lo S.C., Nicholson R.L. (1998). Reduction of light-induced anthocyanin accumulation in inoculated sorghum mesocotyls. *Plant Physiol.*, 116: 979-989.

Davies K. & Winefield C. (2009) Modifying anthocyanin production in flowers**.** Springer Science & Business, 49-84.

Donner H. K, & Robbins T. P. (1991). Genetic and developmental control of anthocyanin biosnthesis. *Ann. Rev. Genet*, 250: 173.

Druka A., Kudrna D., Rostoks N., Brueggeman R., Wettstein D., & Kleinhofs A.

(2003). Chalcone isomerase gene from rice (*Oryza sativa*) and barley (*Hordeum vulgare*): physical, genetic and mutation mapping. *Gene*, 302: 171-178

Feinbaum R.L., & Ausubel F.M. (1988). Transcriptional regulation of the *Arabidopsis thaliana* chalcone synthase gene. *Mol. Cell Biol.*, 6: 1985-1992.

Feinbaum R.L., Storz G., & Ausubel F.M. (1991). High intensity and blue light regulated expression of chimeric chalcone synthase genes in transgenic *Arabidopsis thaliana* plants. *Mol. Gen. Genet.*, 226: 449-56.

Furukawa T., Maekawa M., Oki T., Suda I., Shimada H., Takamure I., & Kadowaki K. (2007). The Rc and Rd genes are involved in prothanocyanidin synthesis in rice pericarp. *Plant J.*, 49: 91-102.

Gollop R., Even S., Colova-Tsolova V., & Peri A. (2002). Expression of the grape dihydroflavonol reductase gene and analysis of its promoter region. *J. Exp. Bot.*, 53: 1397-1409.

Gollop R., Farhi S., & Peri A. (2002). Regulation of the leucoanthocyanidin dioxygenase gene expression in *Vitis vinifera. Plant Sci.*, 161: 579-588.

Guerrero L., Castillo J., Quiñones M., Garcia-Vallvé S., Arola L., Pujadas G., Muguerza B. (2012). Inhibition of Angiotensin-converting enzyme activity by flavonoids: structure-activity relationship studies. *Plos one,* 11: 49493-49504.

Han S.J., Ryu S.N. &Kang S.S. (2004). A New 2-Arylbenzofuran with Antioxidant Activity from the Black Colored Rice (*Oryza sativa* L.) *Bran. Chem. Pharm. Bull.*, 52: 1365-1366.

Harborne J. (1997). Phytochenistry of fruits and vegetables, an ecological overview.

In Tomas-Barberan F, ed, Phytochemistry of Fruit and vegetables. Oxford University press, New York, pp 353-367.

Harborne J., & Williams C. (2000). Advances in flavonoid research science 1992. *Phytochem., 55*: 481-504.

Hiemori M., Koh E., & Mitchell A. E. (2009). Influence of cooking on anthocyanins in black rice (Oryza sativa L. japonica var. SBR). *J. Agric. Food Chem.*, 57: 1908-1914.

Holton T.A., & Cornish E.C. (1995) Genetics and biochemistry of anthocyanin biosynthesis. *Plant Cell*, 7: 1071-1083.

Hu C., Zawistowski J., Ling W. H., & Kitts D.D. (2003). Black rice (*oryza sativa* L. *indica*) Pigmented fraction Suppresses both Reactive Oxygen Species and Nitric Oxide in Chemical and Biological Model System Food chemistry. *J. Agric. Food Chem.*, 51: 5271-5277.

Itani T. (2000). Red rice, purple-black rice and aromatic rice: cultivars, cultivation. Processing and utilixation of Ancient Rice. Nobunkyo, Tokyo, 1-160.

Itani T., Tamaki M., Arai E., & Horino T. (2002). Distribution of amylose, nitrogen, and minerals in rice kernels with various characters. *J. Agric. Food Chem.*, 50: 5326-5332.

Jaakola L., Maatta K., Pirttila A.M., Torronen R., Karenlampi S., & Hohtola A. (2002). Expression of genes involved in Anthocyanin biosynthesis in relation to Anthocyanin, Proanthocyannin, and Flavonol levels during bilberry fruit development. *Plant Physiol.*, 130: 729-739.

Jackson J.A., Fuglevand G., Brown B.A., Shaw M.J., & Jenkins G.I. (1995). Isolation

of Arabidopsis mutants altered in the light-regulation of chalcone synthase gene expression using a transgenic screening approach. *Plant J.*, 8: 369-380.

Jeong S.W., Das P.K., Jeoung S.C., Song J.Y., Lee H.K., Kim Y.K., Kim W.J., Park Y.I., Yoo S.D., Choi S.B., Choi G., & Park Y.I. (2010). Ethylene suppression of sugar-induced anthocyanin pigmentation in *Arabidopsis*. *Plant Physiol.*, 154: 1514-1531.

Jiao Y.J., Ma L.G., Strickland E., & Deng X.W. (2005). Conservation and divergence of light-regulated genome expression pattern during seedling development in rice and *Arabidopsis*. *Plant Cell*, 17: 3239-3256.

John M.C., & Gareth I.J. (1996). Distinct UV-B and UV-A/blue light signal transduction pathways induce chalcone synthesis gene expression in *Arabidopsis* cells. *Plant Cell*, 8: 1555-1567.

Kim M.K., Kim, H., Koh K., Kim H.S., Lee, Y. S., & Kim Y.H. (2008). Identification and quantification of anthocyanin pigments in colored rice. *Nutr. Res. Pract.*, 2: 46-49.

Koide T., Kamei H., Hasimoto Y., Kijoma T., & Haseqawa M. (1996). Antitumor effect of hydrolyzed anthocyanidin from grape rinds and red rice. *Cancer Biother. Radiopham.*, 11: 273-277.

Kubasek W.L., Shirley B.W., Mckillop A., Goodman H.M., Briggs W.B., & Ausubel F.M. (1992). Regulation of flavonoid biosynthetic genes in germinating *Arabidopsis* seedlings. *Plant Cell*, 4: 1229-1236.

Kwon Y., Oh J.E., Noh H, Hong S.W., Bhoo S.H., & Lee H. (2011). The ethylene

signaling pathway has a negative impact on sucrose-induced anthocyanin in *Arabidopsis*. *J. Plant Res.*, 124: 193-200.

Laokuldilok, T., Shoemaker, C. F., Jongkaewwattana, S. R., & Tulyathan V. (2011). Antioxidants and antioxidant activity of several pigmented rice brans. *J. Agric. Food Chem.*, 59: 193-9.

Lazze M. C., Pizzala R., Savio M., Stivala L. A., Prosperi E., & Bianchi L. (2003). Anthocyanins protect against DNA damage induced by tert-butyl-hydroperoxide in rat smooth and hepatoma cells. *Mutat Res.*, 1: 101-115.

Lee J., He K., Stolc V., Lee H., Figueroa P., Gao Y., Tongprasit W., Zhao H., Lee I., & Deng X.W. (2007). Analysis of transcription factor HY5 genomic binding sites revealed its hierarchical role in light regulation of development. *Plant cell*, 19: 731-749.

Lee J.C., Kim J.d., Hsieh F.H., & Eun J.B. (2008). Production of black rice cake using ground black rice and medium-grain brown rice. *Int. J. Food Sci. Tech.*, 43: 1078-1082.

Ling W.H., cheng Q. X., Ma, J., & Wang T. (2001). Red and black rice decrease atherosclerotic plaque formation and increase antioxidant. status in rabbits. *J. Nutr.*, 131: 1421–1426.

Livak K. J, & Schmittgen T. D. (2001). Analysis of relative gene expression data using real-time PCR quantitative PCR and the $2^{-\triangle\triangle Ct}$ method. *Methods*, 25: 402-408.

Lloyd J.C., & Zakhleniuk O.V. (2004). Responses of primary and secondary

metabolism to sugar accumulation revealed by microarray expression analysis of *Arabidopsis* mutant, pho 3. *J. Exp. Bot.*, 55: 1221-1230.

Marles M.A.S., Ray H., & Gruber M.Y. (2003). New perspective on proanthocyanidin biochemistry and molecular regulation. *Phytochem.*, 64: 367-83.

Martinelli E.M., Baj A., & Bombardelli E. (1986) Computer-aided evaluation of liquid-chromatographic profiles for anthocyanins in *Vaccinium myrtillus* fruits. *Anal. Chim. Acta.*, 191: 275–281

McGuire R. G. (1992) Reporting of objective color measurements. *Hort Sci.*, 27: 1254-1255.

Mercier J. (1997). Role of phytoalexins and other antimicrobial compounds from fruits and vegetables in postharvest disease resistance. In Tomas- Barberan F, ed, Phytochemistry of Fruit and Vegetables. Oxford University press, New York Press, New York, pp 221–241.

Mita S., Murano N., Akaike M., & Nakamura K. (1997). Mutants of Arabidopsis thaliana with peliotropic effects on the expression of the gene for β-amylase and on the accumulation of anthocyanin that are inducible by sugars. *Plant J.*, 11: 841-51.

Mol J., Grotewold E., & Koes R. (1998). How genes paint flowers and seeds. *Trends in Plant Science*, 3: 212-217.

Moscovici S., Moalem-Beno D., & Weiss D. (1996). Leaf-mediated light reasponse in petunia flowers. *Plant Physiol.*, 110: 1275-1282.

Nagao S., & Takahashi M. (1963). Trial construction of twelve linkage groups in

Japanese rice. *J. Fac. Agr. Hokkaido Univ.* 53: 72-130.

Nesi N., Jond C., Debeaujon L., Caboche M., & Lepiniec L. (2001). The Arabidopsis TT2 gene encodes a R2R3 MYB domain protein that acts as a key determinant for proanthocyanidin accumulationin developing seed. *Plant Cell*, 13: 2099-2114.

Oki T., Masuda M., Kobayashi M., Nishiba Y., Furuta S., Suda I., & Sato T. (2002). Polymeric Procyanidins as Radical-Scavening Components in Red-Hulled Rice. *J. Agric. Food Chem.*, 50: 7524-7529.

Osawa T., Ramarathnam N., Kawakishi S., & Namiki M. (1992). Phenolic Compounds in Food and Their Effects on health. Antioxidants & Cancer Prevention. Chap.10, ed. By Huang M.-T., Ho C-t., Lee C. Y., American Chemical Society, Washington, pp.122-134.

Pelletier M.K., Burbulis I.E., & Winkel-Shirler B. (1999). Disruption of specific flavonoid genes enhances the accumulation of flavonoid enzymes and end-products in Arabidopsis seedlings. *Plant Mol. Biol.*, 40: 45-54.

Reddy A.M., Reddy V.S., Scheffler B.E., Wienand U., & Reddy A.R. (2007). Novel transgenic rice overexpressing anthocyanidin synthase accumulates a mixture of flavonoids leading to an increased antioxidant potential. *Metab. Eng.*, 9: 95-111.

Reddy A.R., Scheffler B., Madhuri G., Srivastava M.N., Kumar A., Sathyanarayanan P.V., Nair S., & Mohan M. (1996). Chalcone synthase in rice (*Oryza sativa* L.): detection of the CHS protein in seedings and molecular mpping of the chs locus. *Plant Mol. Biol.*, 32: 735-743.

Rice-Evans C.A., Miller N.J., & Paganga G. (1997). Antioxidant properties of phenolic compounds. *Trends in Plant Sci.*, 2: 152-159.

Rolland F., Baena-Gonzalez E., & Sheen J. (2006). Sugar sensing and signaling in plants: conserved and novel mechanisms. *Annu. Rev. Plant Biol.*, 57: 675-709.

Russo A., La Fauci L., Acquaviva R., Campisi A., Raciti G., Scifo C., Renis M., Galvano G., Vanella A., & Galvano F. (2005). Ochratoxin A-induced DNA damage in human fibroblast: protective effect of cyaniding 3-O-beta-d-glucoside. *J. Nutr. Biochem.*, 16: 31-37.

Ryu S.N., Park S.Z. & Ho C.T. (1998). High Performance Liquid Chromatographic Determination of Anthocyanin Pigments in Some Varieties of Black rice. *JFDA.*, 6: 729-736.

Schulze-Lefert P., Dangi J., Becker-Andre M., Hahlbrock K., & Schulz W. (1989). Inducible in vivo DNA footprinting define sequences necessary for UV light activation of the parsley chalcone synthase gene. *EMBO J.*, 8: 651-656.

Shin J., Park E., & Choi G. (2007). PIF3 regulates anthocyanin bisynthesis in an HY5-dependent manner with both factors directly binding anthocyanin biosynthetic gene promoters in Arabidopsis. *Plant J.*, 49: 981-994.

Solfanelli C., Poggi A., Loreti E., Alpi A., & Perata .P (2006). Sucrose-specific induction of the anthocyanin biosynthetic pathway in *Arabidopsis. Plant physiol.*, 140: 637-646.

Sompong R., Siebenhandl-Ehn S., Linsberger-Martin G., & Berghofer E. (2011). Physicochemical and antioxidative properties of red and black rice varieties from

Thailand, China and Sri Lanka. *Food Chem.*, 124: 132-140.

Springob K., Nakajima J., Yamazaki M., & Isaito K. (2003). Recent advances in the biosynthesis and accumulation of anthocyanins. *Nat. Prod. Rep.*, 20: 288-303.

Sultana B., Anwar F., & Przybylski R. (2007). Antioxidant activity of phenolic components present in barks of *Azadirachta india*, *Terminalia arjuna*, *Acacia nilotica*, and *Eugenia jambolana* Lam Trees. *Food Chem.*, 104: 1106-1114.

Sultana B., Anwar F., & Ashraf M. (2009). Effect of extraction solvent/technique on the antioxidant activity of selected medicinal plant extracts. *Molecules*, 14: 2167-2180.

Suzuki M., Kimura T., Yamagishi K., Shinmoto H., & Yamaki K. (2004). Comparison of mineral contents in 8 cultivars of pigmented brown rice. *Nippon Shokuhin Kagaku Kogaku Kaishi*, 51 (8) : 424-427.

Tabart J., Kevers C., Pincemail J., Defraigne J.O., & Dommes J. (2009). Comparative antioxidant capacities of phenolic compounds measured by various tests. *Food Chem.*, 113: 1226-1233.

Tanaka Y., Tsuda S., & Kusumi T. (1998) Physiology metabilic engineering to modify flower color. *Plant Cell*, 11: 1119-1126.

Tian Q. G., Giusti M. M., Stoner G. D., & Schwartz S. J. (2005). Screening for anthocyanins using high-performance liquid chromatography coupled to electrospray ionization tandem mass spectrometry with precursor-ion analysis, product-ion analysis, common-neutral-loss analysis, and selected reaction monitoring. *J. Chromatogr. A.*, 1091 : 72–82.

Toyokuni S., Itani T., Morimitsu Y., Okada K., Ozeki M., Kondo S., Uchida K.,

Osawa T., Hiai H., & Tashiro T. (2002). Protective effect of colored rice over white rice on Fenton reaction-based renal lipid peroxidation in rats. *Free Radic. Res.*, 36: 583-92.

Tsukaya H., Ohsima T., Naito S., Chino M., & Komeda Y. (1991) Sugar-dependent expression of the CHS-A gene for chalcone synthase from petunia in transgenic Arabidopsis. *Plant Physiol.*, 97: 1414-1421.

Vsteen B. (1983). Flavonoids: A class of natural products of high pharmacological potency. *Biochem Pharmacol*, 32: 1141-1148.

Weiss D. (2000). Regulation of flower pigmentation and growth: multiple signalling pathways control anthocyanin synthesis in expanding petals. *Plant Physiol.*, 110: 152-157.

Weisshaar B., & Jenkins G.I. (1998). Phenylpropanoid biosynthesis and its regulation. *Curr. Opin. Plant Biol.*, 3:251-257.

Winkel-Shirley B. (2001). Flavonoid biosynthesis: a colorful model for genetics, biochemistry, cell biology, and biotechnology. *Plant Physiol.*, 126: 485-493.

Wu X., Beecher G. R., Holden J. M., Haytowitz D. B., Gebhardt S. E., & Prior R. L. (2004). Lipophilic and hydrophilic antioxidant capacities of common foods in the United Sates. *J. Agric. Food Chem., 52*: 4026-4037.

Xiaoyun D., Edwards L.B, & Erich G. (2011) Function conservation of plant secondary metabilic enzymes revealed by complementation of Arabidopsis flavonoid mutant with maize genes. *Plant Physiol.*, 127: 46-57.

Yao Y., Sang, W., Zhou M. J. & Ren G. X. (2010). Antioxidant and α-Glucosidase

Activity of Colored Grains in China. *J. Agric. Food Chem., 58*: 770-774.

Zhang M. W., Zhang R. F., Zhang F. X., & Liu R. H. (2010), Phenolic Profiles and Antioxidant Activity of Black Rice Bran of Different Commercially Available Varieties. *J. Agric. Food Chem.*, 58: 7580-7587.

Zhao H. F., Dong J. J., Lu J., Chen J., Li Y., Shan L.J., Lin Y., Fan W., & Gu G.X. (2006). Effects of extraction solvent mixtures on antioxidant activity evaluation and their extraction capacity and selectivity for free phenolic compounds in Barley (*Hordeum vulgare* L.). *J. Agric. Food Chem.*, 54: 7277-7286.

Zhou B., Li Y.H., Xu Z.R., Yan H.F., Homma S., & Kawabta S. (2007). Ultraviolet A-specific induction of anthocyanin biosynthesis in the swollen hypocotyls of turnip. *J. Exp. Bot.*, 58: 1771-1781.

Zhou S., Fang Z., Lu Y., Chen J., Liu D., & Ye X. (2009). Phenolics and antioxidant properties of bayberry (*Myrica rubra Sieb*. et Zucc.) pomace. *Food Chem.*, 113: 884-888.

Acknowledgement

This study was carried out in the laboratory of Hiroshima Prefectural University, from 2009 to 2013. I wish to express my sincere gratitude to many people who have assisted me in the completion of this study on the grounds that your practical advice, critical comments and indispensable encouragement. Foremost among those is my supervisor, Prof. Irifune who provided me more than just technical assistance. Then I am grateful to Prof. Itani, who provide me many help whether living or study. Of course, I am grateful to associate professor Nagao, Prof. Niimi, Prof. Tai, Prof. Oku, Prof. Muto, and Prof. Fujita, Prof. Kohmura and so on. I am grateful to schoolmates of our laboratory, Prof. Itani’s, associate professor Nagao's, and Prof. Niimi's students because they provided me many help. I deeply appreciate all the friends, especially Miss Yuni, Wang Dan and Tuty who provided me many help whether living or spirit, and let me find out feeling of home. Last but not least, I am very grateful to my parents, husband and my son for your understanding and incessant encouragement.

I am gratitude to Japanese government for providing me a scholarship.

Identification and Antioxidant Capacity of Anthocyanin Pigment, and Expressional Analysis of Flavonoid Biosynthetic Genes in Colored Rice Strains

Program in Biological System Science, Graduate School of Comprehensive Scientific Research, Prefectural University of Hiroshima, Japan.