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RESEARCH ARTICLE

Anthocyanin Content in Rice Is Related to Expression Levels of Anthocyanin Biosynthetic Genes

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The composition of anthocyanins was analyzed in two rice cultivars. 'Ilpum' showed no detectable levels, while 'Heugjinju' contained three types of anthocyanins: cyanidin, cyanidin 3-*O*-glucoside, and peonidin 3-*O*-glucoside. We also assessed the expression of anthocyanin biosynthetic genes – for phenylalanine ammonia lyase (*PAL*), chalcone synthase (*CHS*), flavanone 3β-hydroxylase (*F3H*), dihydroflavonol reductase (*DFR*), and anthocyanin synthase (*ANS*) – in different tissues of those cultivars. All five genes were expressed more in the leaves and seeds of 'Heugjinju' than in 'Ilpum', with the greatest amount of transcript being detected in 'Heugjinju' seeds. Two genes, *DFR* and *ANS*, had relatively high expression levels and were specific for anthocyanin biosynthesis. Furthermore, expression of *CHS*, *F3H*, *DFR*, and *ANS* was enhanced during seed maturation and was correlated with ambient temperature during seedling growth.

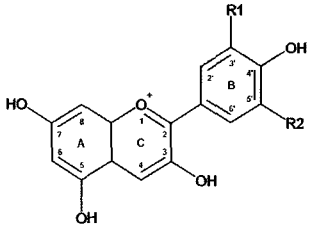
Keywords: anthocyanin biosynthesis, *Oryza sativa*, real-time PCR

Plants contain diverse phenolic compounds that are primarily derived from phenylpropanoid and phenylpropanoid-acetate. The phenylpropanoid pathway is one of the most important for secondary metabolism. Starting from phenylalanine, it directs the biosynthesis of lignins, flavonoids, stilbenes, and coumarins (Holton and Cornish, 1995; Croteau et al., 2000). Lignin, a major constituent of the cell wall, provides structural support. Flavonoids comprise a diverse group of over 6000 compounds; subgroups comprise anthocyanins, proanthocyanidins or condensed tannins, and isoflavonoids. Finally, coumarins and stilbenes have protective roles against bacterial and fungal pathogens.

Recently, plant phenolics have drawn attention because of their benefits to human health (Cornwell et al., 2004). For example, isoflavonoids and stilbenes are known as phytoestrogens, which affect the estrogen-mediated response. In addition, flavonoids can counteract cancer cell growth, as well as promote antioxidant, anti-inflammatory, and vascular activities. Thus, breeders are striving to develop high levels of phenolic compounds in plants. One of the most important of those target compounds is the group of anthocyanins, which constitutes six members: pelargonidin, cyanidin, delphinidin, petunidin, peonidin, and malvidin (Table 1). These are stored as glucosides. These anthocyanins play roles as pigments and UV-B protectants (Springob et al., 2003). In addition to being ingredients of vegetables and fruits, they are considered therapeutic agents that can have an effect on coronary heart disease and certain types of cancers (Ross and Kasum, 2002).

The biosynthetic pathway of anthocyanins has been described in *Arabidopsis* and *Petunia*, and most of the corresponding genes have been cloned. However, their biosynthesis in rice is relatively unknown even though that genus is a model crop plant and its genome has now been sequenced. Nevertheless, some cultivars have been developed that contain high levels of anthocyanins; such efforts are useful in better understanding this biosynthetic pathway in rice. As an initial step, we have now investigated gene expression in two cultivars and examined the effect of temperature on the regulation of anthocyanin biosynthesis.

Table 1. Structure of anthocyanidin.



Anthocyanidins	R1	R2
Delphinidin	OH	OH
Cyanidin	OH	H
Pelargonidin	H	H
Petunidin	OCH_3	OH
Peonidin	OCH_3	H
Malvidin	OCH_3	OCH_3

MATERIALS AND METHODS

Chemicals

Anthocyanin standards, purchased from Polyphenols Laboratories (Norway), included delphinidin 3-*O*-glucoside (Dp-3-glc), delphinidin 3-*O*-rutinoside (Dp-3-rut), cyanidin 3-*O*-glucoside (Cy-3-glc), cyanidin 3,5-*O*-diglucoside (Cy-3,5-diglc), and pelargonidin 3-*O*-glucoside (Pg-3-glc). Ethyl acetate, acetonitrile, and methanol were obtained from Fisher Scientific (USA) while Sigma (USA) was the source of trifluoroacetic acid (TFA).

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Table 2. Primer sets for real-time PCR.

Gene	GenBank accession number	Forward primer	Reverse primer
PAL	X16099	GGTCGTTCCCGCTCTACC	GGAACACCTTGTTGCACTCC
CHS	X89859	GGGATCTCGGACTGGAACTC	CCTCATCCTCTCCTTGTCCA
F3H	XM_474226	AGGAGCCCATACTGGAGGAG	CTCCTTGGCCTTCTTCTTGA
DFR	Y07956	ACATGTTCCCGGAGTACGAC	TACCTGAACCTGAACCCGTG
ANS	Y07955	CTCTCCTGGGTCGTCTTCTG	GTTGCACGTGCTGCTTGAAT
ACT	X16280	ATGAAGATCAAGGTGGTCGC	GTACTCAGCCTTGGCAATCC

Rice Cultivars and Growing Conditions

Plants of two rice cultivars-'Ilpum' and 'Heugjinju' - were grown in a 25-X7-X 2.5-m chamber. Lighting conditions included exposure to full sunlight during the daytime, which was supplemented for 3 h daily with white fluorescent light (400 W) during the Winter. Three different growing temperatures were tested: 21, 24, and 27°C.

Analysis of Anthocyanins

Cyanidin 3-*O*-glucoside chloride, peonidin 3-*O*-glucoside chloride, cyanidin chloride, peonidin chloride, delphinidin chloride, and malvidin chloride were dissolved in a 50:50 water:methanol solution with 0.1% HCl. After the rice seeds were pulverized, 1 g of the powder, also containing 0.5 mg of delphinidin, was used as an internal standard. Extraction was performed twice, each time through continuous shaking for 6 h at 4°C, using 20 mL of the 1:1 water: methanol solution plus 1% HCl. The combined extracts were then centrifuged at 3200 rpm for 20 min, and the supernatants were concentrated with a rotary evaporator at 45°C. A portion of each sample was passed through a 0.45 μm PTFE filter prior to HPLC injection. For our hydrolysis analysis, the supernatants were mixed with a 1:1 solution of water:methanol and 2N HCl, and were hydrolyzed at 100°C for 60 min. The hydrolyzed samples were then immediately cooled to room temperature. These seed extracts were analyzed via HPLC (Series 1100; Agilent Technologies, USA) that was equipped with a photo diode array (PDA) detector and a Phenomenex Luna C18 column (5-um particle size, 4.6 mm × 15.0 cm; Phenomenex, USA). For our analytical scale, the mobile phase consisted of 0.2% TFA, and was programmed as follows: 10% acetonitrile at 0 min, 15% acetonitrile at 20 min, 18% acetonitrile at 30 min, 35% acetonitrile at 50 min, 90% acetonitrile at 60 min, then isotropic from 60 min to 65 min; at a flow rate of 1 mL min^{-1} and detection at 520 nm.

Real-Time Quantitative RT-PCR

Total RNA was isolated from rice tissues using the Plant RNeasy extraction kit (Qiagen, Germany). cDNA was synthesized with a QuantiTect reverse transcription kit (Qiagen) and amplified on a Rotor-Gene RG-3000A thermocycler (Corbett Research, Australia) with a SYBR-GreenR PCR Kit containing Hotstart DNA polymerase (Qiagen). The GenBank accession numbers and primers used here are listed in Table 2. PCR was performed with incubation at 95°C for 15 min to activate the Hotstart Taq DNA polymerase, followed by 45 cycles of 95°C for 10 s, 60°C for 15 s, and 72°C for 20 s. Specificity of these amplification procedures was verified with a heat dissociation protocol. PCR products were sequenced to check the specificity of each primer set. The results obtained from different samples were standardized to the constitutive actin gene. Expression levels for each gene were based on their take-off times, and all experiments were conducted twice.

RESULTS AND DISCUSSION

Determination of Anthocyanin in Two Rice Cultivars

To determine the composition of anthocyanins in our two

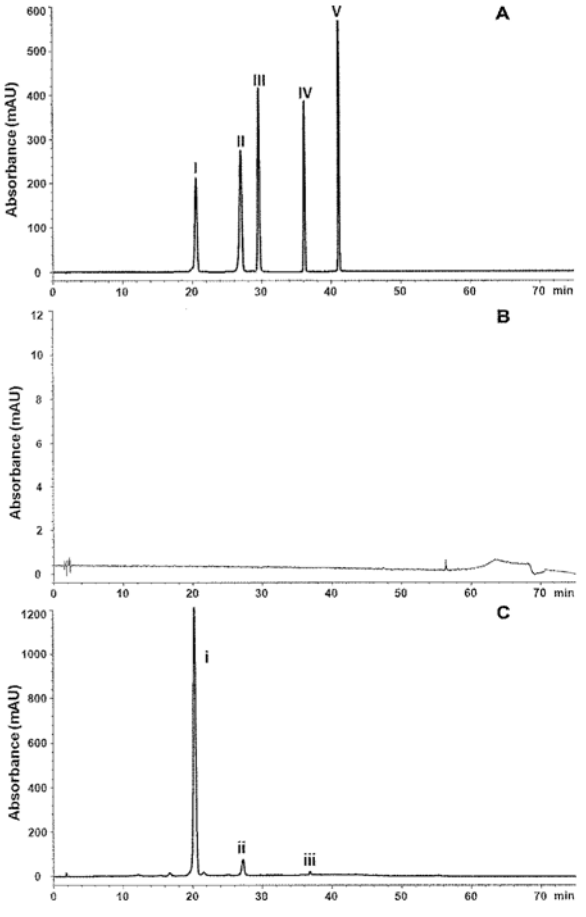

Figure 1. Analysis of anthocyanin composition in 'Ilpum' and 'Heugjinju' rice using HPLC chromatograms of standard mixture, recorded at 520 nm on a C18 column. (**A**) Standard mixture. I, cyanidin-3-*O*-glucoside; II, peondin-3-*O*-glucoside; III, delphinidin; IV, cyanidin; V, peonidin. (**B**) 'Ilpum'. (**C**) 'Heugjinju'. i, cyanidin-3-*O*-glucoside; ii, peniodin-3-glucoside; iii, cyanidin.

rice cultivars, seed extracts were analyzed by HPLC. Product retention times were compared with that of standard anthocyanin (Fig. 1A). The major component of anthocyanin in cv. Heugjinju was cyanidin 3-*O*-glucoside (Fig. 1C), followed by peonidin 3-*O*-glucoside and cyanidin. In contrast, 'Ilpum' showed no detectable anthocyanins (Fig. 1B). Other rice cultivars are known to contain cyanidin 3-*O*-gentiobioside, cyanidin 3-*O*-rhamnoside, cyanidin 3,5-*O*-diglucoside, and cyanidin 3-*O*-rhamnoglucoside, as well as peonidin derivatives such as peonidin 3-*O*-rhamnoglucoside (Ryu et al., 1998; Escribano-Bailón et al., 2004). We also detected some minor peaks but could not identify them because the standard materials were not available. HPLC analysis with the hydrolysates of our extracts revealed only cyanidin and peonidin, indicating that those minor compounds were likely to be derivatives of either component.

Expression Analysis of Anthocyanin Biosynthesis-Related Genes

Five genes were examined for their involvement in anthocyanin biosynthesis: phenylalanine ammonia lyase (*PAL*), chalcone synthase (*CHS*), flavanone 3β-hydroxylase (*F3H*), dihydroflavonol 4-reductase (*DFR*), and anthocyanidin synthase (*ANS*). Among them, PAL is the first enzyme in phenylpropanoid pathways, converting phenylalanine to cinnamic acid and tyrosine to *p*-coumaric acid. Furthermore, CHS catalyzes the first committed step of the flavonoid pathway. The other three -- F3H, DFR, and ANS -- direct the conversion of naringenin into dihydrokaempferol (dihydroflavonol), leucopelargonidin (leucoanthocyanidin), and pelargonidin (anthocyanin).

To investigate possible tissue-specific expression, total RNA was isolated from the roots, leaves, stems, and seeds of 'Ilpum' and 'Heugjinju' at 15 d after flowering. Overall, the expression level for *PAL* was higher than for the other genes in all tissues from both cultivars (Fig. 2). In particular, *PAL* transcripts were greater in the roots and stems. Surprisingly, the highest levels of expression for *CHS* were determined in the stems of 'Ilpum' but also in the seed of 'Heugjinju', while expression of *F3H* was higher in 'Heugjinju' (especially its seed) than in 'Ilpum'. Relatively lower transcript amounts were detected for *DFR* and *ANS*, two important genes in the anthocyanin biosynthetic pathway, with the least being measured from 'Ilpum' seed. This suggests that such low levels may not be sufficient to produce anthocyanin. For all five genes, their expression in seeds was higher in 'Heugjinju' than in 'Ilpum', indicating that the accumulation of anthocyanin is correlated with the expression of genes involved in its biosynthesis. Shimada et al. (2005) also

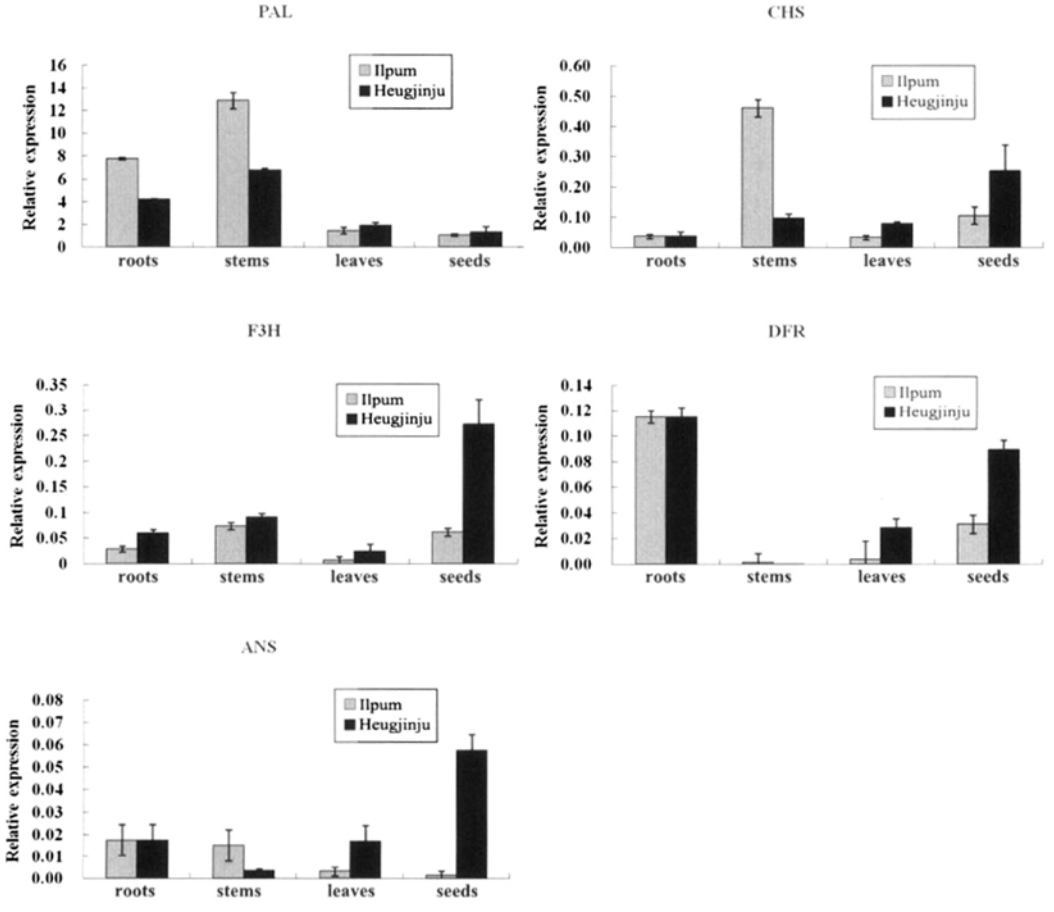

Figure 2. Expression of anthocyanin-biosynthetic genes in two rice cultivars. Total RNA was isolated from roots, stems, leaves, and seeds. Transcript levels are indicated as relative to those from rice actin gene. *PAL*, phenylalanine ammonia lyase; *CHS*, chalcone synthase; *F3H*, flavanone 3β-hydroxylase; *DFR*, dihydroflavanol reductase *ANS*, anthocyanin synthase.

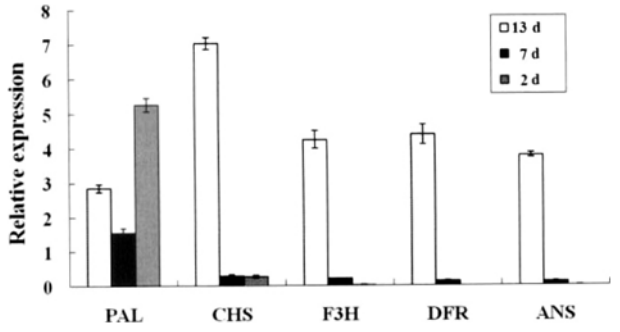

Figure 3. Expression of anthocyanin-biosynthetic genes at 2, 7, and 13 d after flowering, using total RNA isolated from seeds.

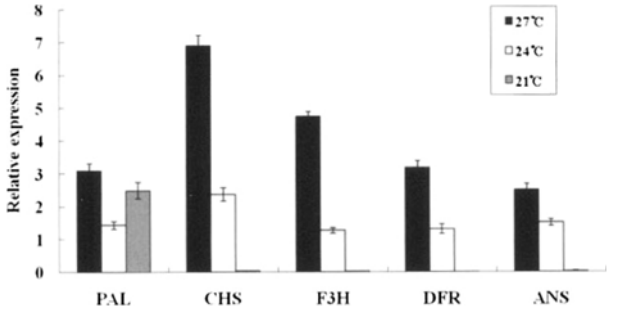

Figure 4. Effects of temperature on expression of anthocyanin-biosynthetic genes. Total RNA was isolated from seeds of rice grown at 21, 24, or 27°C, and collected at 16 d after flowering.

have reported that tissue-specific expression of *DFR* and *ANS* is associated with anthocyanin accumulation. In contrast, *PAL* expression was relatively higher in stems and roots than in leaves or seeds, an observation supported by the involvement of this gene in not only the flavonoid pathway but also the biosynthesis of lignin. Raes et al. (2003) have also demonstrated that *Arabidopsis PAL1* and *PAL2* are more highly expressed in stems and roots compared with other tissues.

Because we had found that anthocyanins were accumulated in the seeds of 'Heugjinju', we decided to investigate the transcript levels for those biosynthetic genes during seed ripening (at 2, 7, and 13 d after flowering). Expression of *CHS*, *F3H*, *DFR*, and *ANS* was enhanced dramatically (24-, 129-, 293-, and 234-fold, respectively) as the seed matured (Fig. 3). Therefore, we might again conclude that the accumulation of anthocyanin is related to the expression of genes involved in its biosynthesis.

We also monitored the effect of temperature on the expression of anthocyanin-biosynthetic genes in 'Heugjinju'. Plants were grown at 21, 24, or 27°C, and their seeds (kernels) were harvested at 16 d after flowering. Although all five genes were expressed, transcript levels were 200- to 500-fold lower for *CHS*, *F3H*, *DFR*, and *ANS* in seeds from plants grown at 21°C compared with our results for those treated at 27°C (Fig. 4). In contrast, *PAL* transcripts were not as strongly influenced by temperature. Finally, the amount of total anthocyanin in 'Heugjinju' grown at 21°C was about one-third of that from seeds of plants grown at 27°C.

Biosynthesis of anthocyanin is regulated in two ways. First, uridine diphosphate-glucose:flavonoid 3-*O*-glucosyltransferase (UFGT) plays an important role that has been demonstrated through a mutation in a regulatory gene from white grapes, which results in the suppression of UFGT expression (Kobayashi et al., 2002, 2004). However, a UFGT homologue that assists in the transfer of a glucose group to anthocyanin has not yet been found in rice (Bowles et al., 2005). Second, *DFR* and *ANS* expression can be suppressed, as has been found in *Caryophyllales* (Shimada et al., 2005). In rice, expression of *DFR* and *ANS* in 'Ilpum' seed was so low that its expression could not be detected on a regular RNA blot. Although it is difficult to draw any conclusion without having a rice UFGT, we might suggest that such expression is critical to anthocyanin accumulation in rice.

Specific transcription factors that regulate flavonoid and anthocyanin biosynthesis have been found in maize, *Arabidopsis*, and *Petunia*. An R2R3-type MYB exhibiting DNA-binding ability and protein-protein interaction has been shown to be involved in this biosynthesis (Nesi et al., 2000; Spelt et al., 2002). These MYB proteins interact with a basic helix-loop-helix (bHLH) protein and, for example, activation of the anthocyanin pathway in maize depends on this relationship between the MYB and bHLH proteins. Thus, one active gene from each family is necessary for flavonoid and anthocyanin biosynthesis (Koes et al., 2005). In rice, the production of anthocyanin is probably mediated by specific transcription regulators. Shin et al. (2006) have reported that overexpression of maize C1, an MYB protein, results in increased flavonoid content in rice. That would suggest a possible corresponding bHLH in rice. In fact, bHLH genes that induce anthocyanin pathway have already been cloned (Sakamoto et al., 2001), but it is still unclear whether these genes for MYB and bHLH interact with each other. In addition, we have identified bHLH genes and MYB family genes that are expressed more actively in 'Heugjinju' rice (unpublished result). Furthermore, one of those, *RCL*, shows high homology with *GL3* in *A. thaliana*, where it is involved in trichome initiation and flavonoid biosynthesis (Zhang et al., 2003). *GL3* also interacts with several rice MYB proteins. Whether this phenomenon contributes to anthocyanin production is currently under investigation.

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LITERATURE CITED

Bowles D, Lsayenkova J, Lim EK, Poppenberger B (2005) Glucosyltransferases: Managers of small molecules. Curr Opin Plant Biol 8: B254-263

Cornwell T, Cohick W, Raskin I (2004) Dietary phytoestrogens and health. Phytochemistry 65: 995-1016

Croteau R, Kutchan TM, Lewis NG (2000) Natural products (sec-

ondary metabolites), *In* BB Buchanan, W Gruissem, RL Jones, eds, Biochemistry and Molecular Biology of Plants. American Society of Plant Physiologists, Rockville, pp 1250-1318

Escribano-Bailón MT, Santos-Buelga C, Rivas-Gonzalo JC (2004) Anthocyanins in cereals. J Chromatog A 1054: 129-141

Holton TA, Cornish EC (1995) Genetics and biochemistry of anthocyanins biosynthesis. Plant Cell 7: 1071-1083

Kobayashi S, Gotto-Yomamotto N, Hirochika H (2004) Retrotransposon-induced mutations in grape skin color. Science 304: 982

Kobayashi S, Ishimaru M, Hiraoka K, Honda C (2002) *Myb*-related genes of the Kyoho grape (*Vitis labruscana*) regulate anthocyanin biosynthesis. Planta 215: 924-933

Koes R, Verweij W, Quattrocchio F (2005) Flavonoids: A colorful model for the regulation and evolution of biochemical pathway. Trends Plant Sci 10: 236-242

Nesi N, Debeaujon I, Jond C, Pelletier G, Caboche M, Lepiniec L (2000) The TT8 gene encodes a basic helix-loop-helix domain protein required for expression of DFR and BAN genes in *Arabidopsis* siliques. Plant Cell 12: 1863-1878

Raes J, Rohde A, Christensen JH, van de Peer Y, Boerjan W (2003) Genome-wide characterization of the lignification toolbox in *Arabidopsis*. Plant Physiol 133: 1051-1071

Ross JA, Kasum CM (2002) Dietary flavonoids: Bioavailability, metabolic effects, and safety. Annu Rev Nutr 22: 19-34

Ryu SN, Park SZ, Ho CT (1998) Liquid chromatographic determination of anthocyanin pigments in some varieties of black rice. J Food Drug Anal 6: 729-736

Sakamoto W, Ohmori T, Kageyama K, Miyazaki C, Saito A, Murata M, Noda K, Maekawa M (2001) The purple leaf (*Pl*) locus of rice: The *Plw* allele has a complex organization and includes two genes encoding basic helix-loop-helix proteins involved in anthocyanin biosynthesis. Plant Cell Physiol 42: 982-991

Shimada S, Inoue YT, Sakuta M (2005) Anthocyanidin synthase in non-anthocyanin-producing *Caryophyllale*s species. Plant J 44: 950-959

Shin YM, Park HJ, Yim SD, Baek NI, Lee CH, An G, Woo YM (2006) Transgenic rice lines expressing maize C1and R-S regulatory genes produce various flavonoids in the endosperm. Plant Biotechnol J 4: 303-315

Spelt C, Quattrocchio F, Mol J, Koes R (2002) ANTHOCYANIN1 of petunia controls pigment synthesis, vacuolar pH, and seed coat development by genetically distinct mechanisms. Plant Cell 14: 2121-2135

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

Zhang F, Gonzales A, Zhao M, Payne CT, Lloyd A (2003) A network of redundant bHLH proteins functions in all TTG1-dependent pathways of *Arabidopsis*. Development 130: 4859-4869