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Expression of multi-copy flavonoid pathway genes coincides with anthocyanin, flavonol and flavan-3-ol accumulation of grapevine

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Summary

The biosynthetic pathways of main grape flavonoids: anthocyanins, flavonols, and flavan-3-ols, hold in common the early step enzymes of biosynthetic pathway: chalcone synthase (CHS), chalcone isomerase (CHI), and flavanone 3-hydroxylase (F3H), the genes of which are multi-copied in the grape genome. The ratios of mRNA levels of the three *Chs*s (*Chs1*, *Chs2*, and *Chs3*), as well as those of the two *Chi*s (*Chi1* and *Chi2*) and those of the two *F3h*s (*F3h1* and *F3h2*), were different among organs, even though no organ-specificity was observed in the strictest sense. Multiple regression analysis demonstrated that the transcription of particular genes significantly coincided with the biosynthesis of a particular flavonoid: the transcription of *Chi2* coincided with flavan-3-ol; *Chs1*, *Chs2*, *F3h1*, and *F3h2* with flavonol; and *Chs2*, *Chs3*, and *F3h2* with anthocyanin biosynthesis. Thus, the transcription of these multi-copy genes is likely induced differently for the biosyntheses of anthocyanins, flavonols, and flavan-3-ols.

K e y w o r d s : flavonoid, chalcone synthase, chalcone isomerase, flavanone 3-hydroxylase.

Introduction

T h r e e c l a s s e s o f f l a v o n o i d s : anthocyanins, flavonols, and flavan-3-ols (monomers and polymeric proanthocyanidins), usually account for 80 to 90 % of the phenolic contents of conventionally produced red wines and contribute to the olfactory profile (Zoecklein *et al*. 1995). Anthocyanins are important components for the color of red wine and table grapes. Flavonols have bitterness and contribute to wine color through copigmentation with anthocyanins (Baranac *et al*. 1997). Flavan-3-ols are also responsible for wine taste: Flavan-3-ol monomers (e.g. (+)-catechin, (–)-epicatechin) have bitterness, and polymeric proanthocyanidins, also known as condensed tannin, contribute to astringency rather than bitterness (Cheynier *et al*. 2006). The three classes of flavonoid compounds are contained in the berry skins, while the seed contains only flavan-3-ols, and various grape organs, e.g. leaves and flowers, contain both of flavan-3-ols and flavonols (Boss *et al*. 1996, Souquet *et al*. 2000). Previous studies showed that the three classes of flavonoids accumulated in organs during different stages of development: anthocyanin accumulated in the berry skins during ripening; flavonol accumulated in the berry skins during the early stage of development and during ripening and also in the leaves, tendrils, and flowers (Downey *et al*. 2003); and flavan-3-ols accumulated in all of these organs and also in the seeds (Boss *et al*. 1996). In addition, the influences of light and plant hormones on flavonol accumulations in berry skins were different from those on anthocyanin accumulations (Fujita *et al*. 2006). These results indicate that the biosyntheses of the three flavonoids are probably controlled differently.

In the flavonoid biosynthetic pathway, chalcone synthase (CHS), chalcone isomerase (CHI), and flavanone 3-hydroxylase (F3H) are held in common in the biosynthesis of anthocyanins, flavonols, and flavan-3-ols (Fig. 1, reviewed by Holton and Cornish 1995). The first key enzyme of flavonoid biosynthesis is CHS, which catalyzes

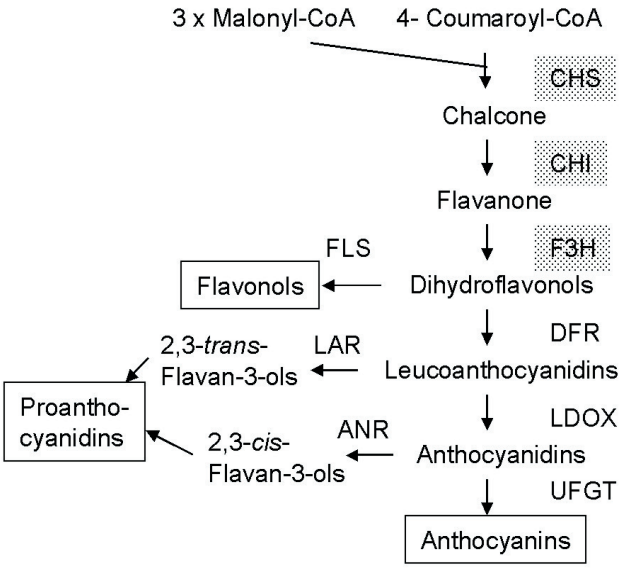

Fig. 1: Simplified biosynthetic pathway of the main flavonoids in grapes. The enzyme names are abbreviated as follows: ANR, anthocyanidin reductase; CHI, chalcone isomerase; CHS, chalcone synthase; DFR, dihydroflavonol 4-reductase; F3H, flavanone 3-hydroxylase; FLS, flavonol synthase; LAR, leucoanthocyanidin reductase; LDOX, leucoanthocyanidin dioxygenase; UFGT, UDP-glucose: flavonoid 3-*O*-glucosyltransferase. The products in boxes and genes in shadow were analyzed in this study.

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the polyketide condensation reaction of *p*-coumaroyl-CoA and three acetate units from malonyl-CoA to yield chalcone. Chalcone is isomerized to flavanone by CHI. Hydroxylation of flavanone at the 3 position by F3H leads to the formation of dihydroflavonol. From dihydroflavonol, the biosynthetic pathway branches to the specific pathway of flavonol, from leucoanthocyanidin to 2,3-*trans* flavan-3-ol, and from anthocyanidin to 2,3-*cis* flavan-3-ol and anthocyanin (Fig. 1). Flavan-3-ol monomers are currently presumed to be the precursors of proanthocyanidins, although several models have been proposed for the proanthocyanidin biosynthesis (Xie and Dixon 2005, Xie *et al*. 2006).

For the expression of the common flavonoid pathway genes in grapes, Boss *et al*. (1996) reported from the result of northern blot analysis that the expression of these genes correlated with the accumulation of anthocyanins and proanthocyanidins in grape berries. In grapes, the three upstream enzymes are encoded by multi-copy genes (Sparvoli *et al*. 1994): three copies of *Chs*s (*Chs1* (Accession no. AB015872), *Chs2* (AB066275), and *Chs3* (AB066274)), two copies of *Chi*s (*Chi1* (X75963) and *Chi2* (TC51784)), and two copies of *F3h*s (*F3h1* (X75965) and *F3h2* (TC45490)) were reported (Jeong *et al*. 2004), where TC number represents a consensus sequence assembly in an expressed sequence tag (EST) database of grape, DFCI Grape Gene Index, Release 5.0 (http://compbio.dfci.harvard.edu/tgi/cgi-bin/tgi/gimain.pl?gudb=grape). Previously, using real-time quantitative-polymerase chain reaction (Q-PCR), we showed that there were significant differences in the mRNA levels among three *Chs*s, between two *Chi*s, and between two *F3h*s during the coloration of grape berry skins (Jeong *et al*. 2004). Similarly, reverse transcription PCR analysis showed that the ratios of mRNA levels in berry skins at the ripening stage and in young leaves were different among the three *Chs*s (Goto-Yamamoto *et al*. 2002). In other plants, phenylalanine ammonia-lyase (PAL) and CHS gene families have been reported to be transcribed organ-specifically (Yamada *et al*. 1992, Kobayashi *et al*. 1998).

Thus, it is important to know how the transcription of each isogene of *Chs*s, *Chi*s, and *F3h*s is controlled depending on the biosynthesis of each flavonoid in each organ in order to understand the control mechanism of flavonoid biosynthesis. We suggest three possibilities: (1) non-specific transcription, e.g. three *Chs*s are transcribed at the same rate in all organs; (2) organ-specific transcription, e.g. *Chs1* is transcribed in leaves, *Chs2* in seeds, and *Chs3* in berry skins; (3) pathway-specific transcription, e.g. *Chs1* is transcribed for flavonol, *Chs2* for flavan-3-ols, and *Chs3* for anthocyanin synthesis, although the results of previous studies showed that the possibility of (1) is unlikely.

In this study, we determined the mRNA levels of all *Chs*, *Chi* and *F3h* isogenes and the concentrations of anthocyanins, flavonols and flavan-3-ols in various grape organs. The ratio of mRNA level of each isogene was different among grape organs, and multiple regression analysis revealed that the transcription of particular isogenes significantly coincided with the biosynthesis of a particular flavonoid.

Material and Methods

Plant material: Four-year-old *Vitis vinifera* 'Cabernet Sauvignon' grown in an experimental vineyard at Higashi-Hiroshima, Japan was used for this study. Flowers at full bloom (June 6, 2003), adventitious roots from cut shoots, young stems of current shoots, young tendrils, small young leaves (reddish green, 4-8 cm in length), middle-size leaves (green, 10-14 cm in length), as well as seeds, berry skins, and flesh at the small-berry stage (2 weeks after flowering, WAF), at the pre-veraison (veraison = the onset of ripening) stage (6 WAF), and at the harvest stage (13 WAF) were sampled. These samples were immediately frozen in liquid nitrogen and kept at -80 ºC until use. The frozen sample was crushed for homogenization before use.

Flavonoid analyses: The flavonoids in grape organs were extracted by the method of Keller and Hrazdina (1998) with slight modifications. Frozen sample (0.2-0.5 g) was ground with a mortar and pestle and extracted with 5-10 ml of extract solution (formic acid:water: methanol = 2:28:70) for 2 h in a dark room. The extracts were centrifuged at 1,600 × *g* for 10 min and then the supernatants were diluted appropriately for each flavonoid analysis. The anthocyanin concentration was determined by reverse-phase HPLC as previously reported (Jeong *et al*. 2006) and expressed as a milligram of malvidin-3-glucoside (Extrasynthese, France) equivalent per gram of fresh weight. HPLC analysis of flavonols was carried out as reported by Koyama *et al*. (2007). The flavonol concentration was expressed as a milligram of quercetin (Fluka, Switzerland) equivalent per gram of fresh weight. The concentration of flavan-3-ols (monomers and proanthocyanidins) was determined by the acidic vanillin method (Sun *et al*. 1988) and was expressed as a milligram of catechin (Sigma, USA) equivalent per gram of fresh weight.

RNA extraction and quantification: Total RNA was extracted from the grape organs as reported by Geuna *et al*. (1998) and treated with RNase-free DNase I (Takara, Japan) to remove contaminant DNA. For the determination of the mRNA levels of *Chs*s (*Chs1*, *Chs2*, and *Chs3*), *Chi*s (*Chi1* and *Chi2*), and *F3h*s (*F3h1* and *F3h2*), Q-PCR was performed using a GeneAmp 5700 sequence detection system (Applied Biosystems, USA) and the QuantiTect SYBR Green PCR Kit (Qiagen, Germany) as described in the manufacturers' manuals. Preparation procedures of sample cDNA and standard DNA for a calibration curve, as well as the sequences of primer sets, except for the reverse primer for *Chi2*, were reported previously (Jeong *et al*. 2004). Q-PCR primers were designed at the low-homology regions of the cDNA sequences of each gene family. The specificity of each primer pair was confirmed by direct sequencing of the amplicon. The sequence of *Chi2* reverse primer was GAAATAAGAGCCTCAAAGAA, of which nucleotide underlined is different from the previous report (Jeong *et al*. 2004). Real-time Q-PCR was performed under the following conditions: 95 ºC for 15 min followed by 35 - 40 cycles at 95 ºC for 15 s, at the annealing temperature of 52 ºC for *Chs1* and 56 ºC for the other genes tested for 20 s, and at 72 ºC for 20 s. The final

primer concentration was 0.25 µM for *Chs1* and 0.5 µM for the other genes tested. Real-time Q-PCR was carried out with at least three replicates per sample, and the average mRNA level of each gene was expressed as a molar ratio relative to *VvUbiquitin1* with reference to Downey *et al*. (2003). The primer sequences and Q-PCR condition for *VvUbiquitin1* were reported by Fujita *et al*. (2005).

Statistics: Multiple linear regression analysis was applied to identify significant independent predictors for mRNA levels of each gene using Microsoft Excel software (Microsoft Office 2000).

Results

Flavonoid accumulation in various grape organs: The concentrations of anthocyanins, flavonols, and flavan-3-ols were determined (Fig. 2). As expected, anthocyanins were detected only in young leaves (reddish green) and berry skin at the harvest stage. Flavonols accumulated most highly in the leaf samples and also were detected in flowers, stems, tendrils, and berry skins, which is consistent with the report of Downey *et al*. (2003). Flavan-3-ols accumulated most highly in the seed samples and also were detected in flowers, roots, young and middle-size leaves, and berry skins. The flavan-3-ol concentration in seeds was already at a high level at the small-berry stage and remained at high levels during berry ripening. The berry skins contained the highest level of flavan-3-ols at the small-berry stage, and their concentration decreased with maturity. With regard to flavan-3-ol biosynthesis in grapes, cloning and expression analyses of leucoanthocyanidin reductase (LAR) and anthocyanin reductase (ANR) genes as well as flavan-3-ol accumulation were reported recently (Fujita *et al*. 2005, 2007, Bogs *et al*. 2006). The change of flavan-3-ol accumulation in this study is consistent with the results of these reports. Although the berry flesh at the small-berry stage (2 WAF) accumulated small quantities of flavan-3-ols, the accumulation was not detectable during berry ripening, which is in agreement with the result reported by Boss *et al*. (1996).

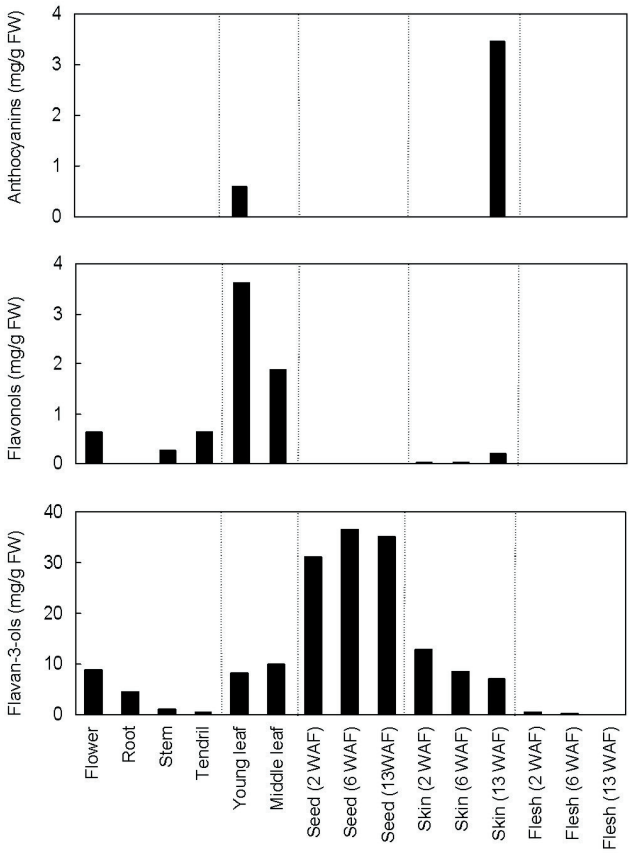

Fig. 2: Accumulation of flavonoids in various grape organs. The concentrations were shown per fresh weight (FW).

Thus, the flavonoid accumulation analyzed here showed no contradiction to other reports, which means these samples have the typical characteristics of red-wine grapes.

mRNA levels of *Chs*s, *Chi*s, and *F3h*s in various grape organs: The mRNA levels of *Chs*s, *Chi*s, and *F3h*s were determined in various grape organs (Fig. 3) using the same samples as that used for the flavonoid analyses. In the leaf samples, the mRNA of all genes tested except for *Chi2*, was detected at higher levels in the young leaves than in the middle-size leaves. In the seed samples, the mRNA of the all genes except *F3h2* showed the highest levels at the small-berry stage (2 WAF). In the berry skin samples, the mRNA of all genes tested was detected at the small-berry stage (2 WAF) and decreased or disappeared at the pre-veraison stage (6 WAF); the mRNA levels increased again at the harvest stage (13 WAF) as was reported by Boss *et al*. (1996). The transcript levels of the three *Chs*s, *Chi2*, and *F3h2* were also high in the flowers, roots, stems, and/or tendrils, as well as in the berry flesh at the small-berry (2 WAF) and harvest (13 WAF) stages.

Of the *Chs*s, the mRNA of *Chs1* and *Chs2* was detected at higher levels in the young leaves than in the other organs tested, whereas the mRNA of *Chs3* was detected at the highest level in the berry skin at the harvest stage (13 WAF). Of the *Chi*s, the mRNA of *Chi2* was detected at a higher level than that of *Chi1* in all of the organs tested. The mRNA of *Chi2* was detected at high levels in the flower and small-berry skins and seeds (2 WAF). The mRNA of *F3h1* was detected at the highest level in the young leaves, whereas the mRNA of *F3h2* was detected at the highest level in the skins at the harvest stage (13 WAF).

Discussion

The mRNA accumulation patterns of total *Chs*s, *Chi*s, and *F3h*s in various grape organs were consistent with the total flavonoid accumulations. In the leaves, which accumulated high levels of flavonols and flavan-3-ols, high levels of mRNA were detected at the young stage. Similarly, in the seeds, which accumulated high levels of flavan-3-ols, high levels of mRNA of these genes were detected at the early stage of development. The berry flesh is an exception, because the mRNA of *F3h2* was accumulated at the harvest stage in spite of the negligible levels of flavonoids. Further work is necessary to clarify this discrepancy.

The mRNA ratios of the three and the two isogenes of *Chs* and *F3h* families, respectively, were different among

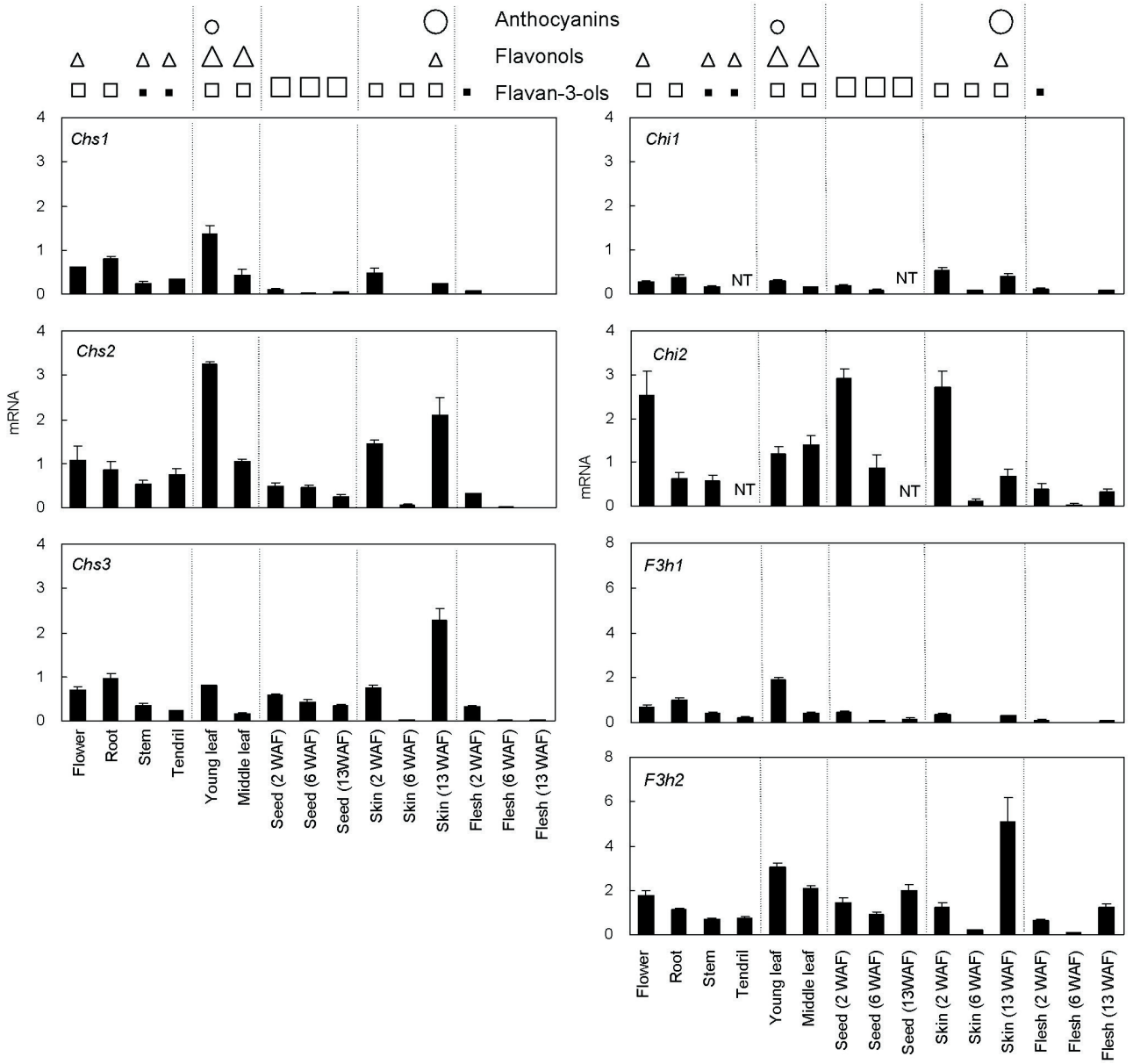

Fig. 3: mRNA levels of *Chs*s, *Chi*s, and *F3h*s expressed as molar ratios to *VvUbiquitin1* in various grape organs. Vertical bars represent standard deviations (n=3). NT, not tested. Simplified accumulation patterns of three classes of flavonoids are shown above the graphs. The sizes of symbols correspond to the relative contents of each flavonoid.

the organs, e.g., the mRNA ratios of *Chs3* and *F3h2* were higher in the berry skins at the ripening stage than in the other samples. The mRNA of these two genes, however, was detected also in other organs. Thus, strictly speaking, no organ-specificity of *Chs*s or *F3h*s expression could be observed.

In the case of *Chi*s, *Chi2* was always predominant at least in mRNA levels although the mRNA ratios were different among the organs. This result is consistent with the frequencies of EST in DFCI Grape Gene Index (Release 5.0), where TC55034 (*Chi1*) consists of 19 ESTs and TC51784 (*Chi2*) consists of 122 ESTs. Previously, we reported that the mRNA levels of *Chi2* were lower than those of *Chi1* in the berry skins (JEONG *et al*. 2004), which must be caused by one base mismatch between the former *Chi2* reverse primer and the corresponding sequence of 'Cabernet Sauvignon'.

To roughly estimate the possibility of the pathway-specific expression, multiple regression analysis was carried out due to the fact that many organs contained two or three classes of flavonoids and simple correlation analysis would not be adequate. The mRNA levels of each gene were used as the criterion variables, with the concentrations of anthocyanins, flavonols and flavan-3-ols as the predictor variables (Table). Strictly speaking, the biosynthesized amount of each flavonoid during a certain period should be used as the predictor variable. These data, however, were not available, and the concentration of each flavonoid was used in stead of them. The data of middle-size leaves, seeds at 6 and 13 WAF, and skins at 6 WAF were omitted from the analysis because these samples showed a decrease or only a small increase in flavonoid concentrations compared to the former stage. Except for *Chi1*, all the tested genes obtained relatively high (from 0.666 to 0.943)

T a b l e

Multiple regression analysis between mRNA levels of multi-copy genes of flavonoid pathway (criterion variables) and concentrations of flavonoids (predictor variables)

	R^2	*t* value		
		Anthocyanins	Flavonols	Flavan-3-ols
Chs1	0.666	-0.27	3.72**	0.33
Chs2	0.859	3.14*	5.32**	1.10
Chs3	0.826	5.54**	0.10	1.42
Chi1	0.253	1.06	0.28	0.88
Chi2	0.718	-0.76	0.22	3.82**
F3h1	0.759	-0.28	4.55**	1.07
F3h2	0.943	9.71**	3.32*	2.17

R^2: contribution ratio, *t*: partial correlation coefficient/standard error, * and ** indicate significance at $p < 0.05$ and $p < 0.01$, respectively.

contribution ratios (R^2) from the accumulation of flavonoids, which suggests that the expressions of these genes were highly related to the accumulation of flavonoids. For the expression of *Chs*s, anthocyanin accumulation showed significantly high *t* values (partial correlation coefficient/standard error) to *Chs2* and *Chs3*, as well as flavonol accumulation to *Chs1* and *Chs2*. Similarly, anthocyanin accumulation showed a significantly high *t* value to *F3h2*, as well as flavonol accumulation to both *F3h1* and *F3h2*. On the other hand, flavan-3-ol accumulation showed significantly high *t* value only to *Chi2*. From these results, it is presumed that the transcriptions of *Chs3* and *F3h2*, and *Chs2* to a lower extent, are related to anthocyanin biosynthesis, even though the transcription of these genes was not biosynthetic-pathway specific in the strictest sense. Similarly, the transcription of *Chs1*, *Chs2*, and *F3h1*, and *F3h2* to a lower extent, are presumed to be correlated to flavonol biosynthesis; and *Chi2* to flavan-3-ol biosynthesis.

The intense coincidence of *Chs2* transcription with flavonol biosynthesis is in accordance with the fact that cDNA of *Chs2* (AF020709) was isolated as a UV-B inducible *Chs* first and that sun light exposure strongly induced flavonol accumulation in grape skin (Price *et al*. 1995, Spayd *et al*. 2002). It was reported that flavonols provide light-resistancy and radical-scavenging ability to higher plants (Nakajima *et al*. 2003, Robak and Gryglewski 1998).

For the biosynthesis of anthocyanin, Ageorges *et al*. (2006) reported that the expression of *Chs3*, glutathione *S*-transferase (*GST*), *UFGT*, and caffeoyl methyl transferase (*CaOMT*), as well as other five non-flavonoid pathway genes, was consistently associated with the colored berry tissues from the results of subtraction analysis and oligonucleotide microarray analysis of red and white grapes. The intense coincidence of *Chs3* transcription with anthocyanin accumulation found here agrees well with their report.

In addition, this new approach of statistical analysis between gene expression levels and product accumulations found possible relations of *F3h1* to flavonol biosynthesis, *F3h2* to anthocyanin, *Chi2* to flavan-3-ols. Thus, for anthocyanin biosynthesis, for example, *Chs3*, *F3h2*, and downstream single-copy genes are highly possibly induced coordinately, and the transcription of the three *Chs*s, as well as those of the two *F3h*s and those of two *Chi*s, are most likely induced differently during the biosyntheses of anthocyanins, flavonols, and proanthocyanidins.

It is well known that flavonoid biosynthesis is controlled by three types of transcription regulatory factors: R2R3 MYB, MYC like basic helix-loop-helix, and WD40 proteins (Mol *et al*. 1998). For flavonoid biosynthesis of grape, VvmybA1 (Kobayashi *et al*. 2004, 2005) and VvmybA2 (Walker *et al*. 2007) were reported to control anthocyanin biosynthesis, and VvMYBPA1 controls flavan-3-ol biosynthesis (Bogs *et al*. 2007). They reported that VvMYBPA1 activated the promoter of *LAR1*, *ANR*, as well as *VvF3'5'H1*, *VvLDOX*, and *VvCHI* (= *Chi1*) coordinately, but not *VvUFGT*. Further researches of transcription regulatory factors will provide us more precise information to understand the control mechanism of flavonoid biosynthesis in grapevine.

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