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ORIGINAL ARTICLE

Characterization of phenolic compounds biosynthesized in pink-colored skin of Japanese indigenous *Vitis vinifera* cv. Koshu grape

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Abstract *Vitis vinifera* cv. Koshu is a traditional grape cultivar that has been grown for centuries in Japan. The Koshu grape has pink-colored skin and Koshu wines have slight astringency. We demonstrated for the first time the characterization of hydroxycinnamic acids, flavan-3-ols, and flavonoids in Koshu grape using high-performance liquid chromatography and liquid chromatography–mass spectrometry. The gross weight of phenolic compounds excluding anthocyanins and proanthocyanidins in Koshu grape at harvest was higher than those in Sauvignon Blanc, Chardonnay, and Merlot grapes. In addition, hydroxycinnamic acid and monomeric flavonol contents in Koshu grape were also higher than those in the other grape cultivars. Transcription analysis of cinnamic acid 4-hydroxylase, *p*-coumarate 3-hydroxylase, caffeate methyltransferase, and flavonol synthase genes indicated high accumulation of hydroxycinnamic acids and flavonols in Koshu grape skin compared with the other cultivars. These findings obtained by chemical and molecular approaches partially explained the phenolic characteristics and the peculiar astringency of Koshu grape.

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Introduction

Vitis vinifera cv. Koshu is an indigenous and typical grape cultivar in Japan, and has been categorized into the cluster of oriental cultivars of *V. vinifera* (Goto-Yamamoto 2000). To obtain high-quality Koshu white wine, various enological techniques, such as the improvement of amino acid contents using sur lies (Ari'Izumi et al. 1994), the amelioration of non-flavonoid compounds by barrel fermentation (Yokotsuka et al. 1994), the removal of bitterness by hyperoxidation (Yokotsuka et al. 2005), and the extraction of aromatic precursors by skin contact (Kobayashi et al. 2007), have been developed. Those enological techniques have contributed to establishing the original style of Koshu wines as well as viticultural practices. In regard to varietal aroma in Koshu wines, we previously demonstrated the contribution of 3-mercaptohexan-1-ol and 3-mercaptohexyl acetate in Koshu wines (Kobayashi et al. 2004) as well as Sauvignon Blanc wine (Tominaga et al. 1998). Thereafter, we showed that *S*-glutathionyl and cysteinyl conjugates act as flavor precursors of 3-mercaptohexan-1-ol in Koshu grape, resulting in the improvement of viticultural practices in Koshu grapevines (Kobayashi et al. 2010).

Koshu grapes have different characteristics from European grapes (*Vitis vinifera* L.) that are generally used for winemaking. The fresh berry weight of Koshu grape at harvest is more than twice those of Chardonnay, Sauvignon Blanc, Merlot, and Cabernet Sauvignon, and the grape skin

becomes pink at the end of the ripening period. Differences in skin color depend on the distribution ratio of anthocyanins, such as cyanidin-based and delphinidin-based anthocyanins (Castellarin and Gaspero 2007; Kobayashi et al. 2009a). In addition, Koshu grape juice has a higher total phenolic content than the juices of other white grape cultivars, and Koshu wines are frequently singled out for their bitterness and/or astringency compared with other white wines. Okamura and Watanabe (1981) reported that the contents of caftaric and coutaric acids, which are the tartrate derivatives of caffeic and coumaric acids, respectively, are much higher in Koshu wines than in white wines made of Semillon, Chardonnay, and Riesling grapes. It is considered that the high contents of those compounds in Koshu wines may have affected the taste of Koshu wines (Yokotsuka 1995). Ethyl caprylate also affected the taste of Koshu wines (Iino et al. 2004).

Although the Koshu grape is known for its astringent flavor, the distribution of phenolic compounds in the berry still needs to be completely clarified. An exhaustive molecular approach that used a cDNA microarray failed to completely elucidate the relationship between the accumulation of phenolic compounds and the transcriptional expression of genes related to phenolic compound synthesis in Koshu grape (Kobayashi et al. 2009b). In this study, we demonstrated (1) the distribution of hydroxycinnamic acids, the hydroxyl derivatives of cinnamic acids—some examples of which are the tartrate derivatives of *p*-coutaric acid and fertaric acid, monomeric flavan-3-ols, and flavonols, in grape tissue, (2) the transcription analysis of genes related to hydroxycinnamic acid, anthocyanin, flavan-3-ol, and flavonol biosyntheses in Koshu grape skin, and (3) a hypothetical model of total phenolic composition in Koshu grape skin. Our findings are expected to contribute to the improvement of Koshu wines. In addition, those findings may be useful for modifying the chemical composition of Koshu grape using genetic engineering approaches, including increasing available polyphenolic compounds, decreasing default phenolic flavor compounds, and producing aroma compounds.

Materials and methods

Chemicals

High-performance liquid chromatography (HPLC) grade solvents were utilized for all chromatographic runs. All solutions were prepared by mixing with Milli-Q water and expressed as a percentage (v/v). *p*-Caftaric acid (97%), *p*-coumaric acid (98%), *p*-ferulic acid (99%), (+)-catechin (98%), (−)-epicatechin (97%), (−)-epicatechin gallate (98%), (−)-gallocatechin (98%), (−)-epigallocatechin (95%), quercetin (98%), and myricetin (96%) were obtained from Sigma–Aldrich (Tokyo, Japan). Quercetin-3-galactoside (98%) and quercetin-3-glucoside (98%) were purchased from Tokiwa Phytochemical Co., Ltd. (Sakura, Chiba, Japan).

Plant materials

All grape cultivars were collected from grapevines in vineyards located in Katsunuma, Yamanashi Prefecture, Japan (latitude, 35°39′8″N; longitude, 138°43′52″E; elevation, 396 m). Sauvignon Blanc, Chardonnay, and Koshu grape cultivars were used for white winemaking, while Merlot was used for red winemaking. Similar cultivation practices, such as leaf removal timing and crop yield, were adopted for Sauvignon Blanc, Chardonnay, and Merlot cultivars. The average yield of the grape cultivars was adjusted to 16 clusters per grapevine. Koshu grapevines were grown by shelf-style cultivation (overhead trellis). The average yield of Koshu was adjusted to 300 clusters per grapevine. All berry clusters were collected at 19 weeks post-flowering (wpf) at the end of the ripening period in 2009. 100–150 fresh berries were randomly sampled and used for analysis.

Analysis of total phenolics, total anthocyanins, and total proanthocyanidins in each grape tissue

Skin, pulp, and seed were separated and pulverized in liquid nitrogen. One gram of the pulverized tissue was added to 4 mL of extraction buffer [methanol containing 0.1% (v/v) HCl] and the mixture was macerated at room temperature for 16 h in the dark. The mixture was centrifuged at $830\times g$ for 10 min and the supernatant was filtered through a 0.45 μm cellulose acetate filter (Advantec Toyo, Tokyo, Japan). The filtrate was used for the quantitative analysis of total phenolics, total anthocyanins, and total proanthocyanidins. Total phenolic content was determined by the Folin–Ciocalteu method with slight modification (Singleton and Rossi 1965) and expressed as gallic acid (μg/g of fresh weight). Total anthocyanin content was determined by the method of Ribéreau-Gayon and Stonestreet (1968) and expressed as malvidin-3-glucoside (μg/g of fresh weight). The proanthocyanidin content (monomeric and polymeric flavan-3-ols) was determined by the acidic vanillin assay (Sun et al. 1998) and expressed as epicatechin (μg/g of fresh weight). The gross weight of other phenolics (i.e., hydroxycinnamic acids and flavonols) was measured by subtracting the amounts of total anthocyanins and proanthocyanidins from the amount of total phenolics.

HPLC conditions for hydroxycinnamic acid determination

The quantitative analysis of hydroxycinnamic acids was performed by reversed-phase HPLC (LC-10Avp, Shimadzu, Kyoto, Japan) using a previously described method with slight modification (Baranowski and Nagel 1981). Although phenolic cinnamates exist as *cis* and *trans* isomers, the gross weight of the *cis* and *trans* configurations was determined in this study. *p*-Coutaric acid and fertaric acid are the tartrates of *p*-coumaric acid and ferulic acid, respectively. According to a previous report by Baranowski and Nagel (1981), *p*-coutaric acid and fertaric acid were identified by HPLC analysis. However, we could not calculate the amounts of *p*-coutaric acid and fertaric acid from the HPLC area counts, because we could not obtain their chemical standards for this analysis. Therefore, we estimated the amounts of *p*-coutaric acid and fertaric acid from the standard curves of *p*-coumaric acid and ferulic acid as calculated from the HPLC area counts.

The HPLC conditions were as follows: Capcell Pak C_{18} column (4.6 mm i.d. × 250 mm, 5 μm, Shiseido, Tokyo, Japan); mobile phase solution A, 0.4% (v/v) phosphoric acid; solution B, 80% (v/v) acetonitrile + 20% (v/v) solution A; flow rate, 1.0 mL/min. Linear gradient programs were performed from solution A:solution B = 90:10 to solution A:solution B = 50:50.

LC–MS conditions for monomeric flavan-3-ol and flavonol determination

The quantitative analysis of monomeric flavan-3-ols and flavonols was performed with an LC–MS system (LCMS-2020; Shimadzu) using a previously described method with slight modification (Fulcrand et al. 1999).

HPLC conditions

The conditions for HPLC (CBM-20A, Shimadzu) were as follows: Atlantis T3 column, 3.0 μm, 2.1 mm × 150 mm (Waters, Milford, MA, USA); column oven temperature, 40°C; mobile phase A [distilled water containing 0.2% (v/v) formic acid]; mobile phase B (acetonitrile); and flow rate, 0.2 mL/min. Linear gradient programs were set from 5% of mobile phase B to 80% of mobile phase B over a 30-min period.

MS conditions

All mass spectrometric data were obtained in the electrospray ionization (ESI) mode. The LC–MS conditions for flavan-3-ols and flavonols were set as follows: probe voltage, −3500 V (ESI-negative mode); nebulizing gas flow, 1.5 L/min; drying gas flow, 15 L/min; CDL temperature, 250°C; block heater temperature, 200°C; injection volume, 2 μL. Mass spectra were recorded for each parent ion as follows (0.3 s): (+)-catechin, *m/z* 288.9; epicatechin, *m/z* 288.9; epicatechin gallate, *m/z* 440.9; gallocatechin, *m/z* 304.9; epigallocatechin, *m/z* 304.9; quercetin-3-*O*-galactoside, *m/z* 463.0; quercetin-3-*O*-glucoside, *m/z* 463.0; quercetin-3-*O*-glucuronide, *m/z* 477.0; quercetin, *m/z* 301.0; and myricetin, *m/z* 317.0. We could not calculate the amount of quercetin-3-*O*-glucuronide from the mass spectra and the area count, because we could not obtain the chemical standard of quercetin-3-*O*-glucuronide for this analysis. Therefore, we estimated the amount of quercetin-3-*O*-glucuronide from the standard curve of quercetin-3-*O*-glucoside and the parent mass spectrum as calculated by LC–MS.

RNA extraction

Grape skin was pulverized in liquid nitrogen. One gram of the powder was added to 10 mL of extraction buffer [0.1 M Tris–HCl (pH 9.5), 20 mM EDTA (pH 8.0), 1.4 M sodium chloride, 2% polyvinyl polypyrrolidone, 2% acetyl trimethyl ammonium bromide, and 0.2% *β*-mercaptoethanol] and the suspension was stirred rapidly for 10 min at 60°C. The suspension was extracted twice with an equal volume of chloroform:isoamyl alcohol (24:1, v/v), and this was followed by centrifugation at 830×*g* for 30 min at 20°C. The supernatant was transferred to a new microtube and 2.5 mL of 0.1 M lithium chloride were added to it. The mixture was incubated at 4°C for at least 16 h and centrifuged at 13200×*g* for 10 min at 4°C. After removal of the supernatant, the pellet was dissolved in 1.0 mL of TE buffer. Total RNA was again precipitated by adding 400 μL of 0.1 M lithium chloride followed by incubation at 4°C for 16 h and centrifugation at 13200×*g* for 10 min at 4°C. Finally, total RNA was purified from the pellet using an RNeasy Plant Mini Kit (Qiagen, Hilden, Germany), as described previously (Tesniere and Vayda 1991).

Real-time quantitative RT-PCR

First-strand cDNA was synthesized from 500 ng of total RNA using a PrimeScript RT Reagent Kit (Takara-Bio Inc., Otsu, Shiga, Japan) according to the manufacturer's instructions. Nucleotide sequences of the primers used in this study were as follows: cinnamic acid 4-hydroxylase (*C4H*) primers (5′-ATCCACCGCCACAACCAT-3′ and 5′-GGCCAGAATTATAGCGCAGAA-3′, corresponding to bases 5015–5032 and 5090–5070, GenBank accession no. AM468511, which have high homology to *Parthenocissus henryana C4H*, GenBank accession no. EU440734,

respectively); *p*-coumarate 3-hydroxylase (*C3H*) primers (5′-GCCCATCGTCGGAAACCT-3′ and 5′-CCCGAACC ACACCGATATG-3′, corresponding to bases 121–138 and 222–202, *V. vinifera* hypothetical protein LOC100263633, GenBank accession no. XM_002283302, which have homology to *Populus alba* × *P. grandidentata C3H*, GenBank accession no. EU391631, respectively); 4-coumaroyl CoA ligase (*4CL*) primers (5′-CTGCCGCTGTT CCACATATACTC-3′ and 5′-CCAATCTCGAACTTCT GCATCA-3′, corresponding to bases 775–797 and 869–848, *V. vinifera* hypothetical protein LOC100245991, GenBank accession no. XM_002283302, which have high homology to *Glycine max 4CL3*, GenBank accession no. AF002257, respectively); caffeate methyltransferase (*CMT*) primers (5′-GATCACCAAACGCAACCCAAA-3′ and 5′-AGGGCTGTTGCGGAGAGA-3′, corresponding to bases 303–323 and 402–385, GenBank accession no. AF239740, respectively); chalcone isomerase (*CHI*) primers (5′-GACGGGTCGCCAGTATTCAG-3′ and 5′-GCTTT GGCTTCTGCGTCAGT-3′, corresponding to bases 320–340 and 409–390, GenBank accession no. X75963, respectively); dihydroflavonol 4-reductase (*DFR*) primers (5′-ACCTGGATGACCTCTGCAATG-3′ and 5′-CAAT CGTGGGAGGAGCAAAT-3′, corresponding to bases 730–750 and 814–795, GenBank accession no. X75964, respectively); leucoanthocyanidin dioxygenase (*LDOX*) primers (5′-GCGATATGACCATCTGGCCTAA-3′ and 5′-ATCCCAACCCAAGCGATAGC-3′, corresponding to bases 544–565 and 663–644, GenBank accession no. X75966, respectively); UFGT, UDP glucose-flavonoid 3-*O*-glucosyl transferase (*UFGT*) primers (5′-CTTC TTCAGCACCAGCCAATC-3′ and 5′-AGGCACACCGT CGGAGATAT-3′, corresponding to bases 540–560 and 638–619, GenBank accession no. AB047099, respectively); leucoanthocyanidin reductase (*LAR*) primers (5′-T CAAGCAGCAGCCGAGAAG-3′ and 5′-CGAGAGAAG TGGCGGTGATC-3′, corresponding to bases 1066–1084 and 1165–1146, predicted *V. vinifera* leucoanthocyanidin reductase 2, GenBank accession no. XM 002273879, respectively); anthocyanidin reductase (*ANR*) primers (5′-TGTGCTGCCAATACCAGTGTTC-3′ and 5′-GGGC TTACTCAGTTGGGACTTTG-3′, corresponding to bases 2500–2521 and 2584–2562, GenBank accession no. XM_002271336, respectively); flavonol synthase 4 (*FLS4*) primers (5′-AGGCACACTTTTCCCACGTT-3′ and 5′-A CCCCCTACGAACCCAGAAG-3′, corresponding to bases 2901–2920 and 3000–2981, GenBank accession no. AB092591, respectively); flavonol synthase 5 (*FLS5*) primers (5′-GCATGTCATGGGCTGTGTTT-3′ and 5′-AC TTGGCAGGGTTTGGTTCA-3′, corresponding to bases 2901–2920 and 3000–2981, GenBank accession no. AB213566, respectively); and 18S rRNA primers (5′-CGA AAGCATTTGCCAAGGAT-3′ and 5′-CCTGGTCGG CATCGTTTATG-3′, corresponding to bases 522–541 and 625–606, GenBank accession no. AF207053, respectively). Real-time quantitative reverse transcription PCR (real-time quantitative RT-PCR) was performed using an ABI Prism 7300 real-time PCR system (Applied Biosystems) with SYBR Green detection modules, as previously described (Kobayashi et al. 2009b). Triplicate experiments on three independent samples were performed in a 96-well reaction plate.

Table 1 Total phenolic, anthocyanin, proanthocyanidin, and other phenolic contents in grape tissues

Tissue	Cultivar	Total phenolics[a]	Total anthocyanins[a]	Total proanthocyanidins[a]	Phenylpropanoids and flavonols[a]
Skin	SB	7033 ± 75.2	283.5 ± 2.9	1593 ± 78.6	5157 ± 33.3
	CD	6183 ± 50.1	218.8 ± 16.8	989 ± 57.1	4976 ± 61.9
	KO	12300 ± 102.1	344.1 ± 14.3	2444 ± 34.9	9511 ± 101.9
	ML	19648 ± 241.4	10357 ± 178.9	2675 ± 56.1	6616 ± 82.7
Pulp	SB	606.7 ± 12.6	76.5 ± 0.7	66.8 ± 8.1	463 ± 10.9
	CD	696.7 ± 28.4	113.4 ± 2.9	114.2 ± 6.2	469 ± 22.1
	KO	823.3 ± 27.5	106.3 ± 3.4	117.9 ± 11.3	599 ± 20.6
	ML	880.1 ± 44.4	185.6 ± 6.6	122.9 ± 6.1	572 ± 48.1
Seed	SB	72532 ± 608.9	352.9 ± 27.1	56791 ± 1796.5	15388 ± 435.1
	CD	58612 ± 517.3	253.8 ± 29.5	41890 ± 1590.8	16468 ± 112.5
	KO	85266 ± 1035.6	335.7 ± 25.1	47965 ± 1749.1	36965 ± 645.6
	ML	110832 ± 1818.7	323.1 ± 21.8	80070 ± 4425.1	30439 ± 604.5

Data are shown as mean ± standard deviations of triplicate experiments from three independent samples

SB Sauvignon Blanc, *CD* Chardonnay, *KO* Koshu, *ML* Merlot

[a] μg/g of fresh weight

Results

Distribution of phenolic compounds in Sauvignon Blanc, Chardonnay, Koshu, and Merlot grape tissues

Total phenolic, total anthocyanin, and total proanthocyanidin contents in each grape tissue at 19 wpf are summarized in Table 1. A large number of phenolic compounds were detected in Merlot grape skin, and approximately half of them were anthocyanins (Table 1). The pink-colored skin of Koshu grape had a moderate total phenolic content that could be positioned between those of green-yellow- and black-colored skins. Interestingly, the skin, pulp, and seed of Koshu grape had the highest phenolic contents (excluding anthocyanins and proanthocyanidins) among those of the four grape cultivars tested. The total phenolic content, excluding the anthocyanin and proanthocyanidin contents, in Koshu grape skin was 1.84-fold, 1.91-fold, and 1.43-fold of those in the skins of Sauvignon Blanc, Chardonnay, and Merlot, respectively. The contents of those compounds in Koshu seed were also approximately twice those of the other white cultivars. Merlot grape had a high proanthocyanidin content, which is one of the important factors that contributes to the taste of red wines (Table 1). The total proanthocyanidin content in Koshu grape was lower than that in red grape. The results demonstrate that Koshu grape distinctively accumulates hydroxycinnamic acids and flavonols, but not proanthocyanidins, in the skin, pulp, and seed.

Quantitative analysis of hydroxycinnamic acids, flavan-3-ols, and flavonoids in each grape tissue

Among the hydroxycinnamic acids shown in Fig. 1, the *p*-caftaric acid content in Koshu grape skin was 392.8 μg/g of fresh weight, which was the highest among the grape skins tested (Fig. 1a, 7.35-fold of Sauvignon Blanc;

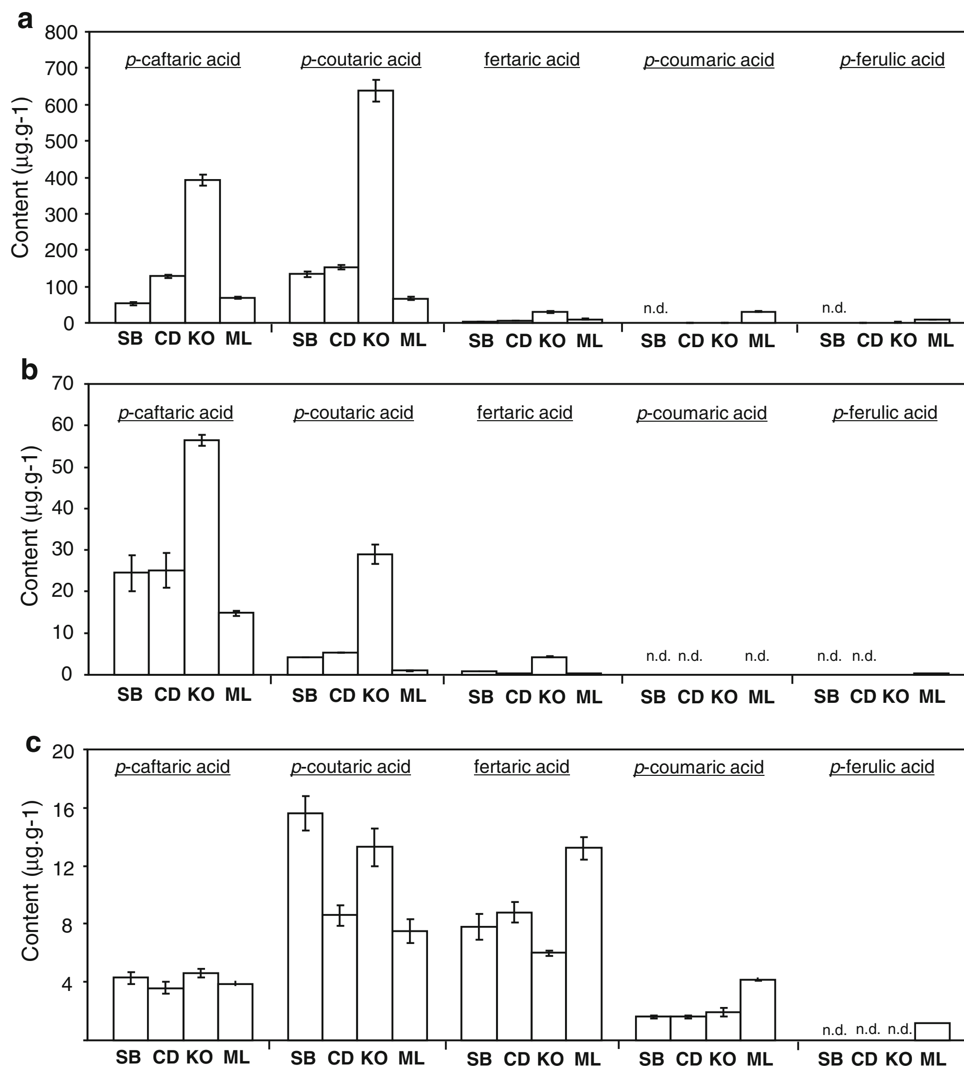

Fig. 1 Hydroxycinnamic acids in four grape cultivars. **a** Skin, **b** pulp, and **c** seed. Grape berries were collected at 19 wpf in the 2009 growing season. *SB* Sauvignon Blanc, *CD* Chardonnay, *KO* Koshu, *ML* Merlot. *n.d.* not detected. Data are shown as the mean ± standard deviation of triplicate experiments from three independent samples

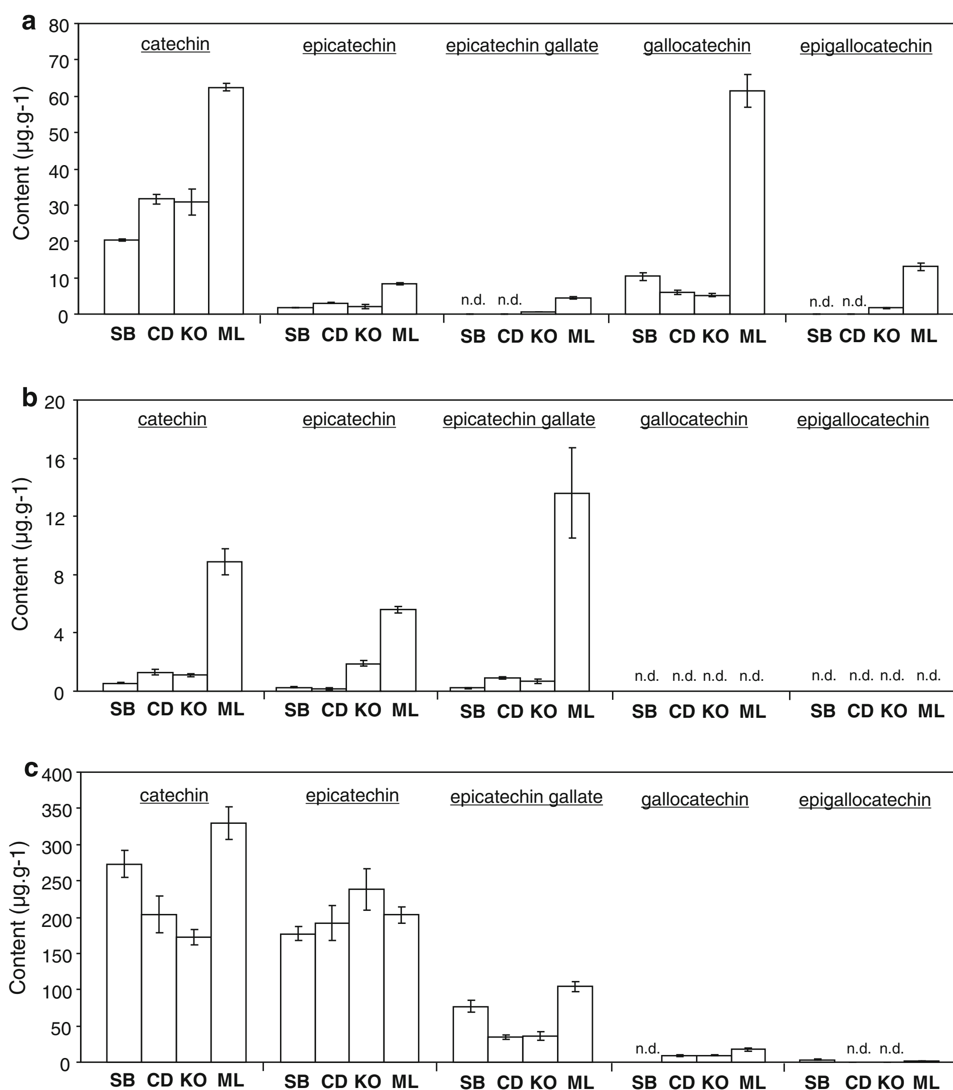

Fig. 2 Monomeric and dimeric flavan-3-ols in four grape cultivars. **a** Skin, **b** pulp, and **c** seed. Grape berries were collected at 19 wpf in 2009 growing season. *SB* Sauvignon Blanc, *CD* Chardonnay, *KO* Koshu, *ML* Merlot, *n.d.* not detected. Data are shown as the mean ± standard deviation of triplicate experiments from three independent samples

3.08-fold of Chardonnay; 5.63-fold of Merlot). Koshu grape skin also contained 639.2 µg of *p*-coutaric acid per gram of fresh weight, which was the highest among the grape skins tested (Fig. 1a, 4.76-fold of Sauvignon Blanc; 4.19-fold of Chardonnay; 9.52-fold of Merlot). In addition to Koshu berry skin, the pulp also had high contents of *p*-coutaric acid and *p*-caftaric acid (Fig. 1b), although the contents were lower than those in the skin. Meanwhile, the hydroxycinnamic acid contents in Koshu seed did not show any distinctive characteristics when compared to the other cultivars (Fig. 1c). The catechin, epicatechin, epicatechin gallate, gallocatechin, and epigallocatechin contents in grape skin, pulp, and seed of Koshu grape were not remarkable (Fig. 2). Merlot grape skin and pulp accumulated much larger amounts of these compounds than the green-yellow- and pink-colored skin cultivars (Fig. 2a, b).

In the flavonols summarized in Fig. 3, the quercetin-3-*O*-galactoside content in Koshu berry skin was 92.7 µg/g of fresh weight, the highest among the grape skins tested (Fig. 3a, 2.34-fold of Sauvignon Blanc; 1.30-fold of Chardonnay; 2.13-fold of Merlot). Koshu grape skin accumulated more quercetin-3-*O*-glucoside than the other grape skins, with the content being 500.6 µg/g of the fresh weight (Fig. 3a, 1.45-fold of Sauvignon Blanc; 1.39-fold of Chardonnay; 1.80-fold of Merlot). In addition, the quercetin-3-*O*-glucuronide content in Koshu grape skin was also higher than those in the other grape skins, 330.5 µg/g of the fresh weight (Fig. 3a, 2.11-fold of Sauvignon Blanc; 5.63-fold of Chardonnay; 1.78-fold of Merlot). Quercetin-3-*O*-glucoside and quercetin were detected in Koshu pulp (Fig. 3b), although their contents were lower than those in the skins (Fig. 3a). Quercetin-3-*O*-glucoside content was the highest in Koshu seed (Fig. 3c).

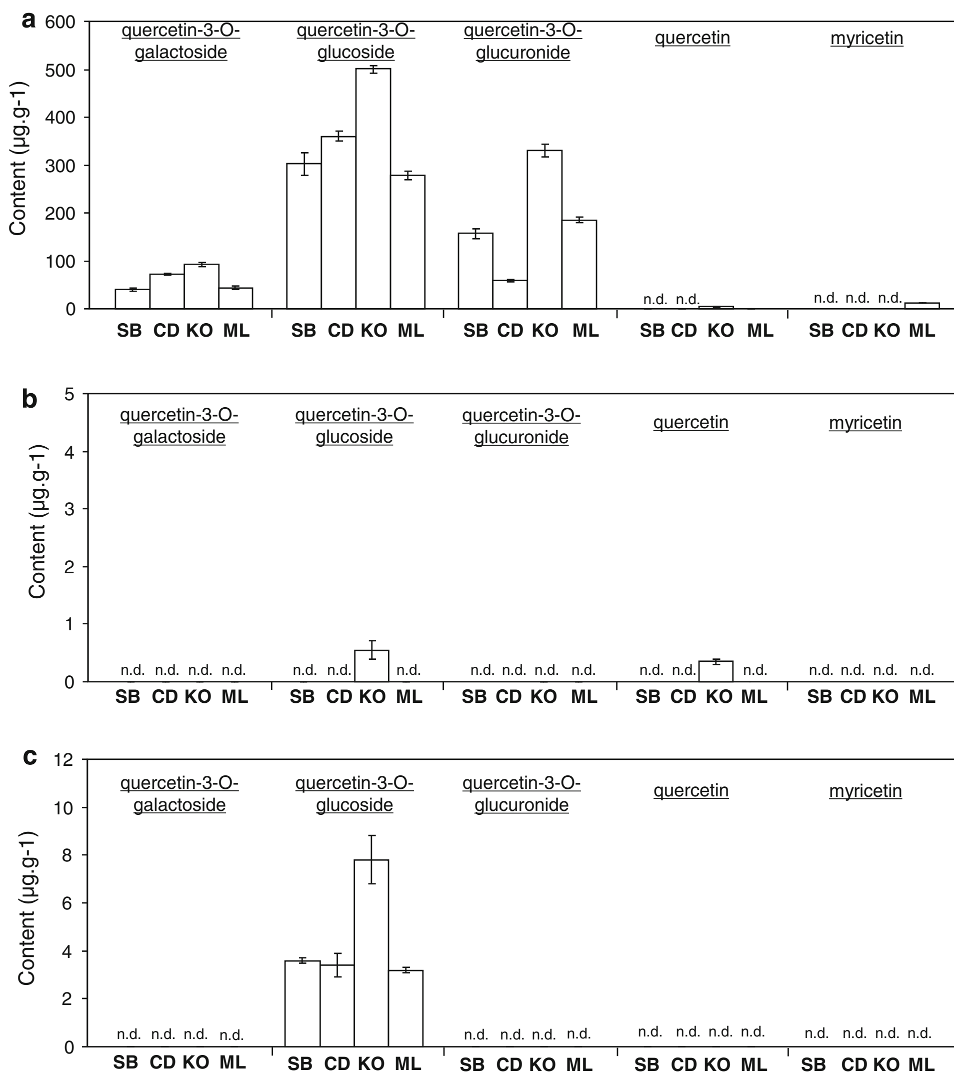

Fig. 3 Monomeric flavonoids in four grape cultivars. **a** Skin, **b** pulp, and **c** seed. Grape berries were collected at 19 wpf in 2009 growing season. *SB* Sauvignon Blanc, *CD* Chardonnay, *KO* Koshu, *ML* Merlot, *n.d.* not detected. Data are shown as the mean ± standard deviation of triplicate experiments from three independent samples

Transcription analysis of genes related to the synthesis of hydroxycinnamic acids, flavan-3-ols, and flavonoids in Koshu grape skin

To understand the accumulation of hydroxycinnamic acids and flavonols in Koshu grape, we performed the transcription analysis of twelve genes, *C4H*, *C3H*, *4CL*, *CMT*, *CHI*, *DFR*, *LDOX*, *UFGT*, *LAR*, *ANR*, *FLS4*, and *FLS5*, in Sauvignon Blanc, Chardonnay, Koshu, and Merlot grape skins (Fig. 4a). The transcripts of genes related to hydroxycinnamic acid synthesis, such as *C4H*, *C3H*, and *CMT*, were abundantly expressed in Koshu grape skin compared with the other grape skins (Fig. 4b, c, e, respectively). These results supported the observation that Koshu grape accumulated large amounts of hydroxycinnamic acids in the skin (Table 1; Fig. 1). The expression of the *CHI*, *DFR*, *LDOX*, and *UFGT* genes (which lead to anthocyanin synthesis) in Koshu skin was lower than that in Merlot grape berry skin, supporting the fact that Koshu grape skin is pink in color (Fig. 4f, g, h, i, respectively). In contrast, Merlot grape skin abundantly expressed the *CHI*, *DFR*, *LDOX*, *UFGT*, *LAR*, and *ANR* genes (Fig. 4f, g, h, i, j, k, respectively). The high expression of *DFR*, *LDOX*, and *UFGT* transcripts in Merlot grape skin contributed to anthocyanin accumulation (Table 1), while the high expression of *LAR* and *ANR* genes (which are related to proanthocyanidin synthesis) induced the accumulation of monomeric and dimeric flavan-3-ols in Merlot grape skin (Table 1; Fig. 2a). A high expression of the *FLS4* and *FLS5* genes (which are related to flavonol synthesis) was detected in Koshu grape skin compared with Sauvignon Blanc, Chardonnay, and Merlot grape skins (Fig. 4l, m, respectively). These results indicate that Koshu grape skin accumulates large amounts of flavonols compared with the other grape skins (Table 1; Fig. 3a), and suggest that this characteristic of Koshu grape skin may be attributed to the

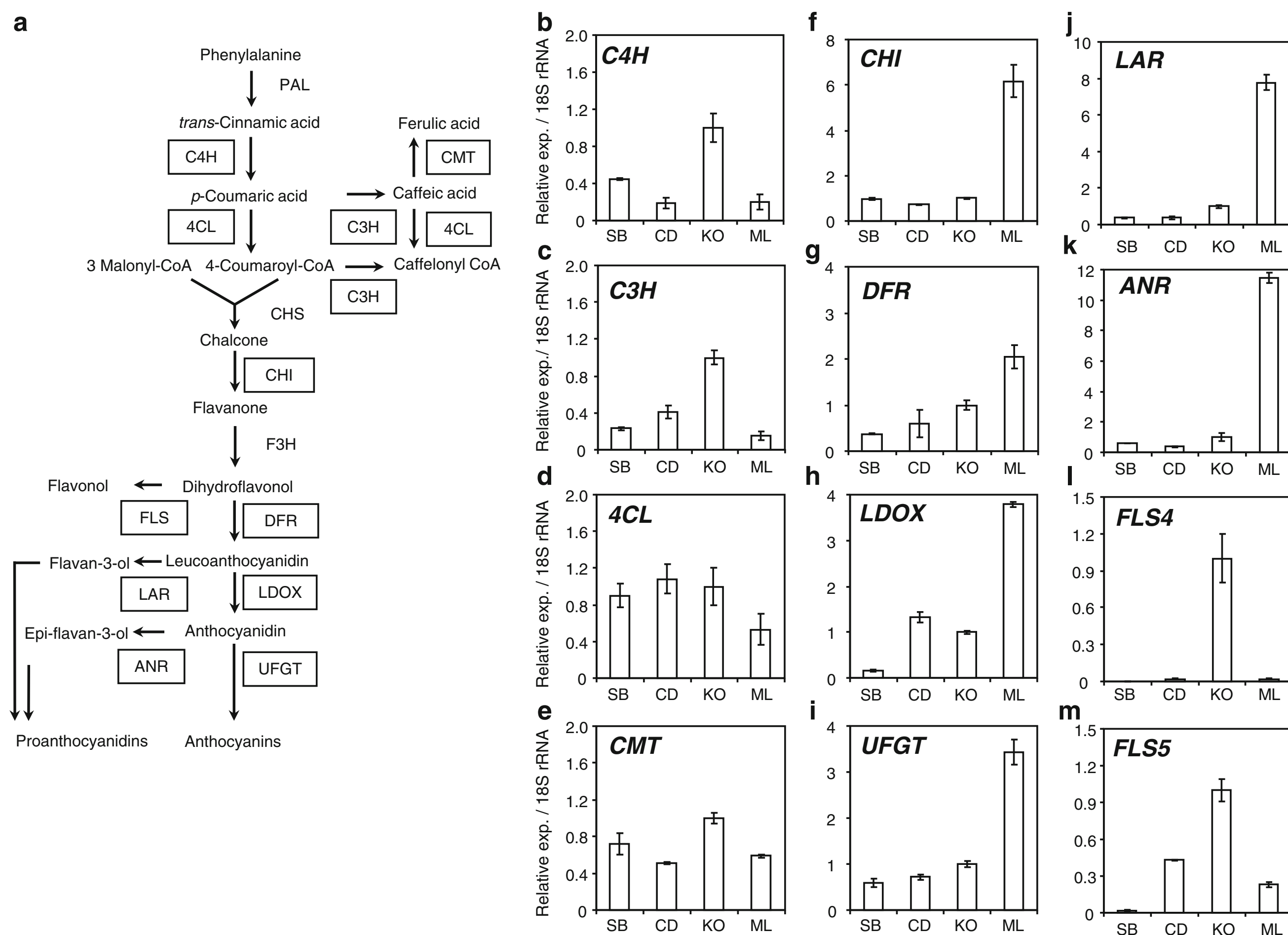

Fig. 4 Transcription analysis of genes related to the synthesis of hydroxycinnamic acids, flavan-3-ols, and flavonoids in Koshu grape skin. **a** Schematic representation of the biosynthetic pathway of hydroxycinnamic acids, anthocyanins, flavan-3-ols, flavonols, and proanthocyanidins. Genes investigated in this study are *enclosed in squares*. *C4H* cinnamic acid 4-hydroxylase, *C3H* *p*-coumarate 3-hydroxylase, *4CL* 4-coumaroyl CoA ligase, *CMT* caffeate methyltransferase, *CHI* chalcone isomerase, *DFR* dihydroflavonol 4-reductase, *LDOX* leucoanthocyanidin dioxygenase, *UFGT* UDP glucose-flavonoid 3-*O*-glucosyl transferase, *FLS* flavonol synthase, *LAR* leucoanthocyanidin reductase, *ANR* anthocyanidin reductase. **b–m** Transcription profiles of the genes. 18S rRNA was used as the internal control. *SB* Sauvignon Blanc, *CD* Chardonnay, *KO* Koshu, *ML* Merlot. Data were calculated as the gene expression relative to 18S rRNA, and gene expression was normalized to that of KO. *Bars* indicate the mean ± standard deviation of triplicate experiments from three independent samples

expression profiles of flavonol synthase genes. *FLS1*, *FLS2*, and *FLS3* transcripts were not detected in the grape skins of any of the cultivars (data not shown).

Discussion

The large amounts of *p*-caftaric and *p*-coutaric acids in Koshu grape skin and pulp (Fig. 1a, b) effectively explained one of the unique characteristics of Koshu juice, from which large amounts of phenols were extracted by high-pressure pressing (Yokotsuka 1990). The transcription profiles of *C4H*, *C3H*, and *CMT* in Koshu grape skin may have contributed to the accumulation of these compounds in Koshu grape skin (Fig. 4b, c, e, respectively). *p*-Coutaric acid is synthesized by the conjugation of *p*-coumaric acid with tartaric acid. In grape berry, *p*-coumaric acid acts as a flavor precursor of volatile phenols, including 4-vinylphenol, a compound responsible for the well-known olfactory default called "phenolic off-flavor" (Chatonnet et al. 1993). Our previous study demonstrated that almost all commercial Koshu wines had high contents of 4-vinylphenol and 4-vinylguaiacol (Kobayashi et al. 2006), and suggested that those compounds might be responsible for the phenolic and smoky aroma in Koshu wines. In addition, the skin contact and/or maceration methods used in Koshu winemaking might extract large amounts of hydroxycinnamic acids from skin into juice, resulting in the strong astringency of Koshu wines (data not shown). Considering the fact that pulp and seed hardly accumulated

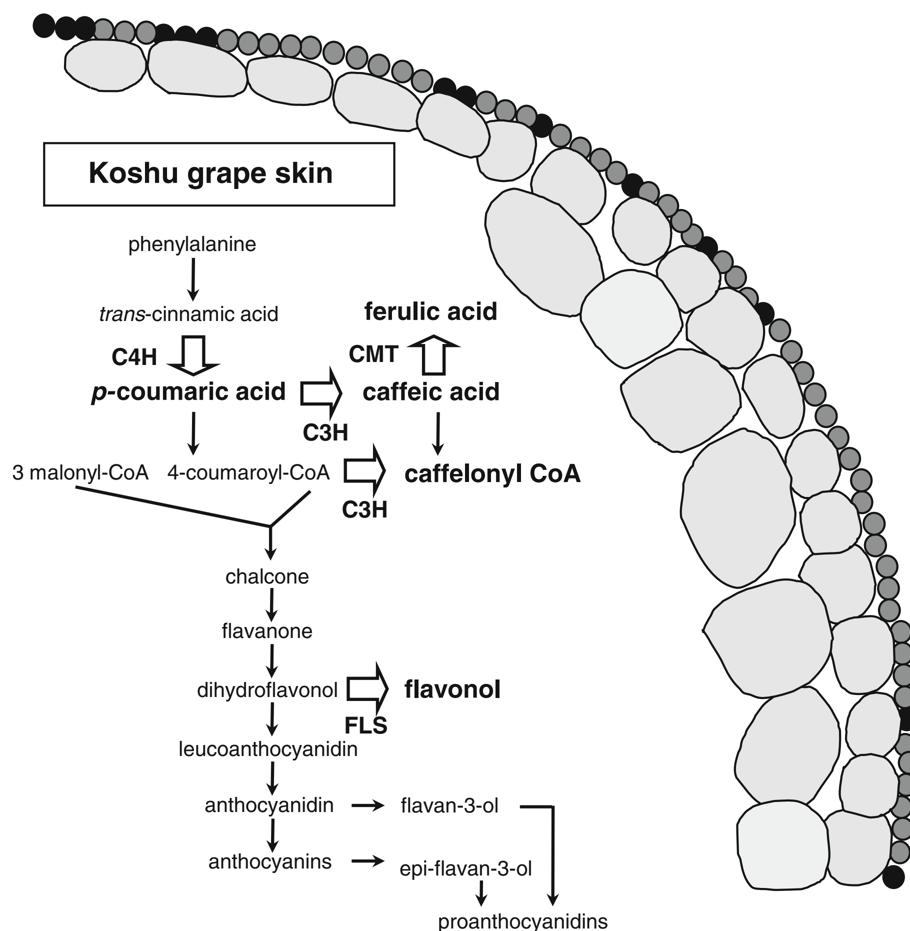

Fig. 5 Hypothetical model of total phenolic composition in Koshu grape skin. *Large arrows* with enzymes in *boldface* indicate high enzymatic activities in the biosynthesis of phenolic compounds in Koshu grape skin. Compounds in *boldface* are phenolic compounds accumulated in Koshu grape skin, as indicated in Table 1 and Figs. 1, 3, and 4

hydroxycinnamic acids, irrespective of the cultivar (Fig. 1), the phenolic aroma and astringency of Koshu wines might be attributable to Koshu grape skin.

Flavonols exist widely in plants and mostly form glucoside conjugates (Cheynier and Rigaud 1986). Flavonols act as a stabilizer of wine color through copigmentation with anthocyanins (Baranac et al. 1997). Koshu grape skin accumulated large amounts of flavonols (Fig. 3). Pink-colored Koshu wines made by long skin contact showed long-term color stability (data not shown). Flavonols were hardly detected in Koshu pulp and seed, as supported by previous reports using other cultivars (Downey et al. 2003; Fujita et al. 2006; Jeong et al. 2008). Flavonol synthase is the key enzyme for the conversion of dihydroflavonols into flavonols. Grape has five genes that encode flavonol synthases, *VvFLS1* to *VvFLS5* (Fujita 2008). *VvFLS1*, *VvFLS2*, and *VvFLS3* were mainly expressed in leaf, bud, and inflorescence, but not in grape skin at harvest (Downey et al. 2003; Fujita 2008). Our transcription analysis of *FLS* genes was consistent with those reports, demonstrating that Koshu grape skin expressed the *FLS4* and *FLS5* genes at 19 wpf (Fig. 4), but not *FLS1*, *FLS2*, and *FLS3* genes (data not shown). Based on these findings, the expression profiles of flavonol synthase genes in Koshu grape skin confer unique characteristics to Koshu grape, enabling it to accumulate large amounts of flavonols in the skin. These compounds affect the bitterness of wine (Koyama et al. 2007), suggesting that the astringency of Koshu wines might be also attributed to flavonoids in Koshu grape skin as well as hydroxycinnamate. The flavonol content of grape skin may be influenced by various viticultural practices, such as vine water status (Kennedy et al. 2002), bunch shading (Downey et al. 2004), and exposure to sunlight (Price et al. 1995; Matus et al. 2009). Thus, canopy management may improve the amount of flavonols in Koshu grape skin and the taste of Koshu wines.

From the above study, we propose a hypothetical model of total phenolic composition in Koshu grape skin (Fig. 5). *p*-Coumaric acid, caffeic acid, and flavonol contents are characteristic of Koshu grape skin and much higher than those in the other grape cultivars. These hydroxycinnamic acids and flavonols may contribute to the taste of Koshu grape and wine, such as its astringency and bitterness. The skin contact method and the maceration method for Koshu winemaking enhance the extraction of the compounds from Koshu grape skin, resulting in the astringency and bitterness of Koshu wines. These findings may serve as a tool to devise new ideas for the development of unique Koshu

wines in the future. In addition, to further explore the characteristics of Koshu grape and wine by chemical and molecular approaches, critical analyses of alkaloids and terpenoids, which are strongly related to bitterness (Dashek 1997), are required.

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