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Polyphenol Composition in the Ripe Fruits of *Fragaria* Species and Transcriptional Analyses of Key Genes in the Pathway

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Supporting Information

ABSTRACT: Polyphenolics are important secondary metabolites in strawberry as they fulfill a wide variety of physiological functions and are beneficial to human health. Seventeen structurally well-defined phenolic compounds including phenylpropanoids, flavonols, flavan-3-ols, and anthocyanins were individually analyzed by LC-MS in the ripe fruits of two cultivars of the commercial strawberry (*Fragaria* × *ananassa* Duch., Rosaceae) as well as in accessions of *F. vesca*, *F. moschata*, and *F. chiloensis*. Metabolic analysis revealed that the majority of the compounds analyzed accumulated in a genotype-dependent manner. Transcriptional studies of genes encoding for enzymes of the biosynthetic pathway such as phenylalanine ammonia-lyase, cinnamic acid 4-hydroxylase, chalcone synthase, and flavonoid 3′-hydroxylase could partially explain the different levels of polyphenolics observed in the *Fragaria* species. The results can provide a sound basis for selecting markers for the development of cultivars with high phenolic content, which can be of value for the food industry.

KEYWORDS: strawberry, ripe fruits, *Fragaria* species, phenolic acid derivatives, flavonols, proanthocyanidins, anthocyanins

INTRODUCTION

Strawberry (*Fragaria* × *ananassa*) fruit is a rich source of polyphenolics,[1] a group of natural secondary metabolites that have been claimed to have a beneficial effect on human health,[2−4] although the importance of antioxidant activity for health aspects has been doubted lately in recent studies.[5] Polyphenolics differ greatly in molecular size, as the group includes very simple structures such as hydroxycinnamic and hydroxybenzoic acids as well as flavonoids and large polymers of high molecular weight such as proanthocyanidins. These compounds are also relevant for other quality parameters of plant-derived foods, such as appearance, taste, and flavor.[6]

Flavonoids are the predominant class of phenolic compounds in plants. They account for approximately two-thirds of the dietary phenols.[7] All of them share the basic skeleton, the flavan nucleus, which consists of two aromatic rings with six carbon atoms (rings A and B) interconnected by a heterocycle that includes three carbon atoms (ring C). According to the modifications of the central C-ring, they can be divided into different structural classes such as flavanones, isoflavones, flavones, flavonols, dihydroflavonols, proanthocyanidins, and anthocyanidins.[8,9]

Previous studies in strawberry have established that the accumulation of phenolic compounds depends on different factors such as genotype,[10] maturation stage,[11] and environmental growth conditions such as climate, irrigation, soil fertility, temperature, cultural systems, harvesting, and handling.[12] The relatively short history of the cultivated strawberry and the breeding programs have produced a small but significant reduction in the genetic diversity of this species[13] and, consequently, has limited the contribution of the genotype to the variability of polyphenolics composition of the ripe fruits. However, information on the polyphenolics content of the fruits of different species of the *Fragaria* genus, which represent a wider genetic background, is limited. Therefore, information on polyphenolic composition of fruits of other species of the *Fragaria* genus, such as *F. vesca*, *F. moschata*, and *F. chiloensis*, can be of interest to study the genetic contribution to the polyphenolic composition.

In this study, five different genotypes belonging to *Fragaria* genus have been analyzed at metabolic and transcriptional levels. The cultivated strawberry *F.* × *ananassa* is an octoploid ($2n = 8x = 56$) that resulted from the natural hybridization between the octoploid species *F. virginiana* and *F. chiloensis*.[14] The wild diploid *F. vesca*, which has been considered to be the model for the genus,[15] is considered to be a subgenome donor in ploidization events.[16] The hexaploid *F. moschata*, or musk strawberry, is known for its intense aroma and superb flavor. In addition to these three genotypes of the *Fragaria* genus, two genotypes of *F.* × *ananassa* (cv. Chandler and cv. Parker) with California pedigrees[13] were selected for this study.

The phenolic compounds here analyzed included phenolic acid derivatives, flavonols, flavan-3-ols, and anthocyanins, all of

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them synthesized in the general phenylpropanoids pathway and seem to be responsible for important organoleptic characteristics of the strawberry fruits.[17] Moreover, a further step has been performed to investigate the influence of genetic variability on the metabolic differences observed. Thus, the mRNA levels of different genes involved in the synthesis of enzymes of the polyphenolic pathway such as phenylalanine ammonia-lyase (PAL), cinnamic acid 4-hydroxylase (C4H), chalcone synthase (CHS), and flavonoid 3′-hydroxylase (F3′H) were determined in the same ripe fruits. The study is supported by the fact that comparison between the transcriptome of *F. vesca* and *F.* × *ananassa* shows differences being quantitative rather than qualitative.[18]

MATERIALS AND METHODS

Plant Material. Amounts of 2 kg of ripe fruits of *F. vesca*, *F. chiloensis*, *F. moschata*, and *F.* × *ananassa* (cv. Camarosa and cv. Parker) were collected from at least six different plants maintained in the *Fragaria* germplasm collection (IFAPA-CIFA Churriana, Málaga, Spain) in 2006. Fully ripe fruits were harvested, selecting for uniformity of size and color, immediately frozen in liquid N_2, and stored at −20 °C until LC-MS and qRT-PCR analyses. Samples of fruits at different developmental stages were from plants (*F.* × *ananassa* Duch. cv. Camarosa) grown under field conditions in Huelva, southwestern Spain. Fruits were harvested at green (green achenes and receptacle), white (green achenes and white receptacle), and red (red achenes and receptacle) stages, immediately frozen in liquid N_2, and stored at −20 °C until qRT-PCR analysis.

Chemicals. Phenylpropenoyl glucose esters were synthesized as described previously.[19] In brief, the strawberry glucosyltransferase *FaGT2* was expressed in *Escherichia coli* and the recombinant enzyme used for the glucosylation of cinnamic acid, 4-coumaric acid, caffeic acid, and syringic acid. Anthocyanins were identified by LC-MS by comparison with the data obtained with commercially available reference material. Proanthocyanins were putatively identified according to the data previously published.[20,21] Pelargonidin 3-*O*-glucoside, quercetin 3-*O*-glucuronide, quercetin 3-*O*-glucoside, kaempferol 3-*O*-glucuronide, kaempferol 3-*O*-glucoside, catechin, epicatechin, 4-methylumbelliferyl glucoside, and biochanin A were obtained from Roth, Karlsruhe, Germany.

Metabolite Analysis. Ripe fruits of different species belonging to the *Fragaria* genus were analyzed by LC-MS. Harvested strawberry fruits were freeze-dried for 72 h and powdered at room temperature using a morter. Approximately 200 mg of powder was dissolved in 250 μL of methanol (containing 12.5 μg of 4-methylumbelliferyl glucoside and 12.5 μg of biochanin A) and centrifuged (16000*g*, 10 min), and the supernatant was used directly for LC-MS analyses.

The system used for metabolite analysis was a Bruker Esquire 3000 Plus mass spectrometer, equipped with an Agilent 1100 HPLC system composed of an Agilent 1100 quaternary pump and an Agilent 1100 variable-wavelength detector. The column was a Eurospher C18 column, particle size = 5 μm, 10 × 2 mm (GromAnalytik & HPLC GmbH, Rottenburg-Halfingen, Germany). The LC parameters went from 0% acetonitrile and 100% water (acidified with 0.05% formic acid) to 50% acetonitrile and 50% acidic water in 50 min, then in 20 min to 100% acetonitrile, held for 10 min at these conditions, and then returned to 100% water and 0% acetonitrile in 5 min at a flow rate of 0.2 mL min^{-1}. The detection wavelength was 280 nm. The ionization parameters were as follows: the voltage of the capillary was 3074 V, and the end plate was set to −500 V. The capillary exit was −109.8 V and the Octopole RF amplitude 120 Vpp. The temperature of the dry gas (N_2) was 300 °C at a flow of 10 L min^{-1}. The full scan mass spectra of the metabolites were measured from *m*/*z* 50−800 until the ICC target reached either 20000 or 200 ms. Tandem mass spectrometry was performed using helium as the collision gas, and the collision energy was set at 1.00 V. Mass spectra were acquired in negative and positive ionization modes. Autotandem mass spectrometry was used to break down the most abundant $[M + H]^+$, $[M - H]^-$, or $[M + HCOO]^-$ ions of the different compounds of the strawberry extracts.

Relative Quantification and Statistical Evaluation of Polyphenolics. Fruit juices obtained from ripe fruits of different species of *Fragaria* genus were analyzed by LC-MS. All fruits were harvested at the same ripening stage. Metabolites were identified by their retention times, mass spectra, and product ion spectra in comparison with the data determined for authentic reference material. Signals of the compounds were integrated in their $[M + H]^+$, $[M - H]^-$, or $[M + HCOO]^-$ ion traces (Supporting Information, Figures S1−S5). The non-natural 4-methylumbelliferyl glucoside was used as internal standard for relative quantification of the metabolites. Moreover, this compound served as control for ionization yield of the mass spectrometer and reproducibility of the retention times. Values are expressed in mg kg^{-1}-equivalent 4-methylumbelliferyl glucoside (dry weight). Statistical significance levels were calculated using the Holm−Sidak method ($p < 0.05$) implemented by SigmaStat software (Supporting Information, Table S1).

Total RNA Extraction and cDNA Synthesis. Total RNA from three independent pools (six to seven strawberry fruits per pool) at different ripening stages, green (about 7 days after anthesis), white (about 14 days after anthesis), and red (about 30 days after anthesis) fruits, in the case of *F.* × *ananassa* (cv. Camarosa), and total RNA from three independent pools of fruits (six to seven strawberry fruits per pool) at ripe stage of *F. vesca*, *F. moschata*, *F. chiloensis*, and *F.* × *ananassa* (cv. Camarosa and cv. Parker) were extracted as described previously.[22] In all cases, the quality of RNA was verified by demonstration of intact ribosomal bands following agarose gel electrophoresis in addition to the absorbance ratios ($A^{280/260}$) of 1.8−2.0. Extracted RNA was treated with amplification grade DNase1 (Sigma-Aldrich, Steinheim, Germany) to remove any DNA contamination prior to cDNA synthesis. Reverse transcription (RT) was carried out using 1 μg of total RNA and the iScript cDNA Synthesis Kit (Bio-Rad, Hercules, CA), following the manufacturer's instructions.

Quantitative Real-Time PCR (qRT-PCR). qRT-PCR analysis was performed using the iCycler system (Bio-Rad) as described previously.[23] Primers for gene-specific amplification (*PAL*, forward, 5′-GAT GCA AAG GCT AAG GCA AG-3′, and reverse, 5′-AGC CCT AAC GCT CTC AAC CT-3′; *C4H*, forward, 5′-GGA CGC TCA ACA GAA AGG AG-3′, and reverse, 5′-TTC ACA AGC TCG GCT ATT CC-3′; *CHS*, forward, 5′-GCC TTT GTT TGA GCT GGT CT-3′, and reverse, 5′-CCC AGG AAC ATC TTT GAG GA-3′; *F3′H*, forward, 5′-GAA GAT CAG CTC CGT CCA TC-3′, and reverse, 5′-CTC TCA GGG CAA AAC TCG TC-3′) were designed using the Primer 3 program (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi) to generate a product of 100−200 bp and to have a T_m (melting temperature) of 60 ± 1 °C and a length of 19−23 bp. PCR reactions were carried out in triplicate. The reaction mix (25 μL per reaction) contained *iQSYBRGreenSupermix 2x*, 10 μM forward and reverse primers, and 1 μL of cDNA of the appropriate dilution, which was selected according to the primer amplification efficiency. The thermal cycling conditions consisted of an initial denaturation step of 94 °C for 3 min, followed by 40 cycles of 94 °C for 30 s, 60 °C for 30 s, and 72 °C for 3 min. The specificity of the PCR amplification was monitored by melting curve analysis following the final step of the PCR, from 65 through 94 at 0.1 °C s^{-1}. PCR efficiencies of all primers were calculated using dilution curves with six dilution points, 2-fold dilution, and the equation $E = [10^{(-1/slope)}] - 1$. The 26S−18S RNA housekeeping gene was used to normalize as endogenous reference (forward, 5′-ACCGTT GAT TCG CAC AAT TGG TCA TCG-3′, and reverse, 5′-TAC TGC GGG TCG GCA ATC GGA CG-3′). Statistical analysis was performed using SigmaStat sofware. Significant differences were determined by

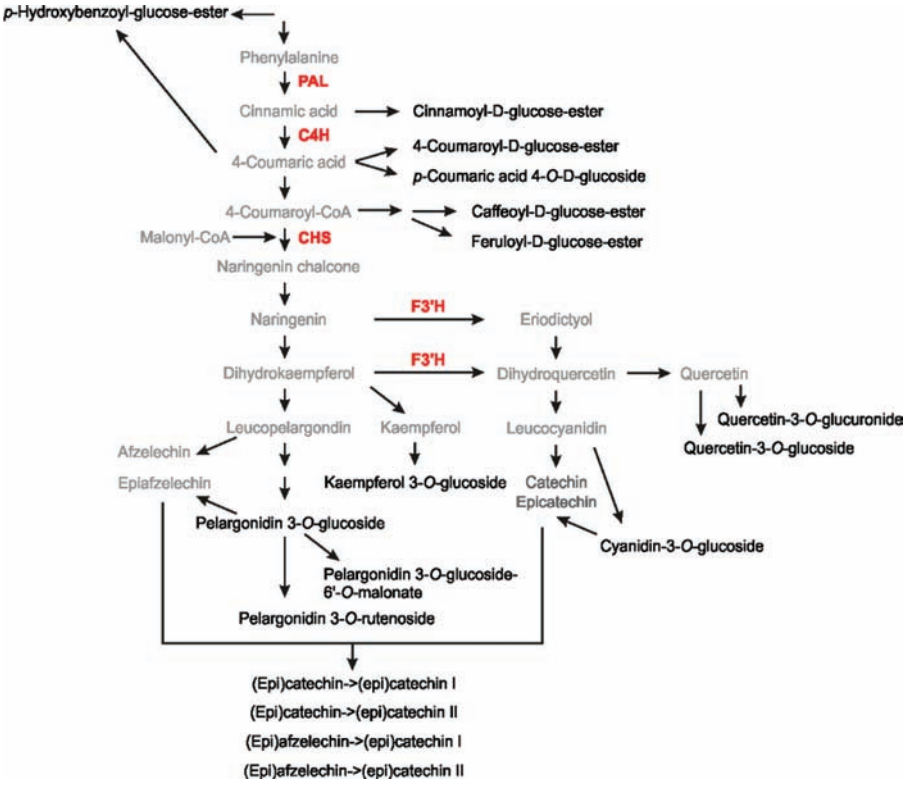

Figure 1. Biosynthetic pathway of polyphenolics: detected metabolites (black), analyzed genes (red), and expected intermediates (gray).

ANOVA. The data are presented as the mean ± SD of three biological replicates, each having three technical replicates in each reaction.

RESULTS AND DISCUSSION

Seventeen phenolic compounds were identified and quantified in the ripe fruits of different species belonging to the genus *Fragaria* (Figure 1). The analyzed compounds can be classified into four different groups: phenolic acid derivatives, flavonols, flavan-3-ols, and anthocyanins. The fruits analyzed were from *F. vesca* (*Fv*), *F. moschata* (*Fm*), *F. chiloensis* (*Fc*), and two commercial cultivars of *F.* × *ananassa*, cv. Camarosa (*Fa*C) and cv. Parker (*Fa*P). The levels of the majority of the analyzed compounds showed a significant genotype dependence in accordance with previous studies.[1,17,24,25] In all cases the major compounds were the anthocyanins pelargonidin 3-*O*-glucoside and cyanidin 3-*O*-glucoside and the phenolic acid derivative cinnamoyl-D-glucose ester. These metabolites play an important role in both color and aroma development of the ripe fruits, which are essential organoleptic characteristics directly related to the quality of the fruits.

Accumulation of Phenolic Acid Derivatives. Previously, studies on the composition of phenolic acid derivatives have been performed on strawberry.[26,27] Their levels show a high variability among the analyzed genotypes (Figure 2). The major compound in all samples was cinnamoyl-D-glucose ester, a metabolite directly related to the formation of methyl and ethyl esters of cinnamic acid, both crucial components of the aroma of the strawberry fruit.[28,29] The fruits of *F. chiloensis* and *F.* × *ananassa* (cv. Parker) contained the highest and lowest levels of this metabolite, respectively. The ripe fruits of *F. vesca*, *F. moschata*, and *F.* × *ananassa* (cv. Camarosa) showed an intermediate content of this compound. The second metabolite in

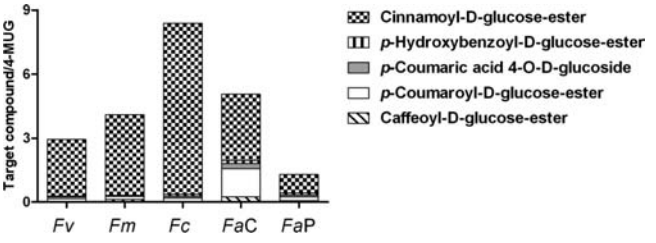

Figure 2. Content of phenolic acid derivatives from ripe fruits of different genotypes of wild and cultivated strawberry. The values shown are the mean of eight independent measurements (mg kg^{-1}-equivalent 4-methylumbelliferyl glucoside). *Fv*, *F. vesca* (Reina de los Valles); *Fm*, *F. moschata* (Caprón Royal); *Fc*, *F. chiloensis* (Red Chiloensis); *FaC*, *F.* × *ananassa* (cv. Camarosa); *Fa*P, *F.* × *ananassa* (cv. Parker).

relative concentration was *p*-coumaroyl-D-glucose ester, the content of which in the ripe fruits of cv. Camarosa of the *F.* × *ananassa* species was 10-fold higher than in the other analyzed fruits. The accumulation of its structural isomer, *p*-coumaric acid 4-*O*-D-glucoside, was comparable in *F. vesca*, *F. chiloensis*, and *F.* × *ananassa* (cv. Parker), whereas the ripe fruits of *F. moschata* and *F.* × *ananassa* (cv. Camarosa) showed the lowest and highest contents of this metabolite, respectively. The concentration of caffeoyl-D-glucose ester was at a maximum in the fruits of cv. Camarosa of *F.* × *ananassa*, which was 10-fold higher than in *F. vesca* and *F. chiloensis*, in which the level of this phenolic acid derivative was the lowest. Finally, in the case of *p*-hydroxybenzoyl-D-glucose ester, again cv. Camarosa of *F.* × *ananassa* contained the highest level in the ripe fruits, followed by *F. chiloensis* and *F.* × *ananassa* (cv. Parker), in which the amount of this compound was similar and higher than in *F. vesca* and *F. moschata*, which accumulated the lowest amounts. Only minor

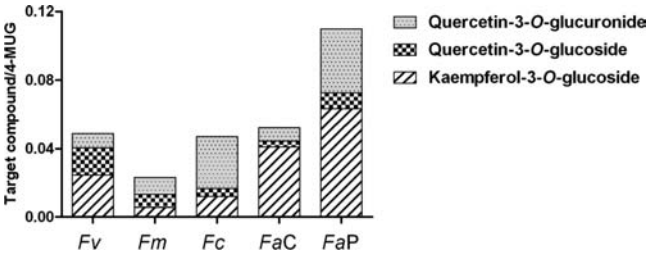

Figure 3. Content of flavonols from ripe fruits of different genotypes of wild and cultivated strawberry. The values shown are the mean of eight independent measurements (mg kg^{-1}-equivalent 4-methylumbelliferyl glucoside). *Fv*, *F. vesca* (Reina de los Valles); *Fm*, *F. moschata* (Caprón Royal); *Fc*, *F. chiloensis* (Red Chiloensis); *Fa*C, *F.* × *ananassa* (cv. Camarosa); *Fa*P, *F.* × *ananassa* (cv. Parker).

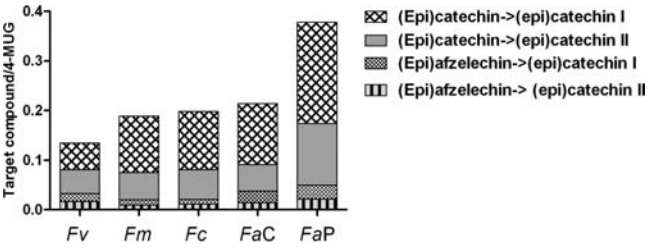

Figure 4. Content of flavan-3-ols from ripe fruits of different genotypes of wild and cultivated strawberry. The values shown are the mean of eight independent measurements (mg kg^{-1}-equivalent 4-methylumbelliferyl-glucoside). *Fv*, *F. vesca* (Reina de los Valles); *Fm*, *F. moschata* (Caprón Royal); *Fc*, *F. chiloensis* (Red Chiloensis); *Fa*C, *F.* × *ananassa* (cv. Camarosa); *Fa*P, *F.* × *ananassa* (cv. Parker).

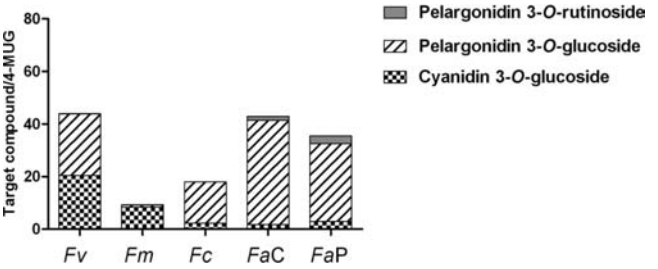

Figure 5. Content of anthocyanins from ripe fruits of different genotypes of wild and cultivated strawberry. The values shown are the mean of eight independent measurements (mg kg^{-1}-equivalent 4-methylumbelliferyl glucoside). *Fv*, *F. vesca* (Reina de los Valles); *Fm*, *F. moschata* (Caprón Royal); *Fc*, *F. chiloensis* (Red Chiloensis); *Fa*C, *F.* × *ananassa* (cv. Camarosa); *Fa*P, *F.* × *ananassa* (cv. Parker).

levels of feruloyl-D-glucose ester were detected as previously described.[30]

The overall comparison revealed that cv. Camarosa of *F.* × *ananassa* accumulated the highest content of all analyzed phenolic acid derivatives at the expense of cinnamoyl-D-glucose ester.

Accumulation of Flavonols. The flavonols have multiple biological functions, including protection against UV light and copigmentation of flowers and fruits.[31] Moreover, these compounds have been reported to possess significant antioxidant properties, and previous studies have reported that an inverse relationship exists between the intake of these compounds and the risk of coronary heart disease,[32] cerebral infarction,[33] and lung cancer.[34] Although their presence is minor in strawberry fruits from a quantitative point of view, as shown by previous paper,[26] interest is focused on flavonols, mainly quercetin and kaempferol derivatives, due to their putative higher bioavailability.[35]

The content of flavonols also presented high variability among the analyzed *Fragaria* species, their overall content being higher in the diploid *F. vesca*, the octoploid *F. chiloensis*, and cv. Camarosa and cv. Parker of *F.* × *ananassa* compared to hexaploid *F. moschata* (Figure 3). It has been reported that flavonoids are synthesized in the cytoplasm and then transported to the vacuoles.[8] Flavonoids only can be accumulated into this organelle in glucosylated form as water-soluble compounds because they are unstable at vacuolar pH.[36] Thus, in accordance with previous studies, kaempferol and quercetin were undetectable in all cases.

Kaempferol 3-*O*-glucoside and quercetin 3-*O*-glucoside were detected in all analyzed genotypes, with higher amounts of kaempferol 3-*O*-glucoside except in the species *F. moschata*, in which the quantity of both metabolites was similar. The kaempferol 3-*O*-glucoside concentration was higher in cv. Camarosa and cv. Parker of *F.* × *ananassa* than in the other species. With regard to quercetin 3-*O*-glucoside, its accumulation was highest in *F. vesca*. Finally, the quercetin 3-*O*-glucuronide concentrations were similar in *F. vesca*, *F. moschata*, and *F.* × *ananassa* (cv. Camarosa), whereas *F. chiloensis* and cv. Parker of *F.* × *ananassa* showed a 3-fold higher content.

Accumulation of Flavan-3-ols. Proanthocyanidins, also known as condensed tannins, are polymeric flavonoids that are thought to be synthesized by sequential addition of intermediates derived from flavan-3,4-diol (e.g., leucocyanidin) to a flavan-3-ol initiating unit (e.g., catechin) or a pre-existing chain.[8] Their presence has been related in different strawberry varieties.[26] In unripe fruits, large amounts of these compounds are accumulated and presumably contribute to the restriction of plant pathogens.[36] They are beneficial owing to their antioxidative capacity, and reports of several in vitro assays demonstrate potentially significant interactions of proanthocyanidins with biological systems, such as antiviral, antibacterial, and radical-scavenging systems.[37]

The level of the proanthocyanidin (epi)catechin→(epi)catechin (isomer I) was similar in all analyzed genotypes with the exception of cv. Parker of *F.* × *ananassa*, in which the quantity of this compound was 2-fold higher (Figure 4). Its structural isomer, (epi)catechin→(epi)catechin (isomer II), was accumulated at lower concentration than isomer I. Only the wild variety *F. vesca* presented a similar content of both isomers. Cv. Parker belonging to the *F.* × *ananassa* species and the diploid *F. vesca* showed the highest and lowest concentrations of (epi)catechin→(epi)catechin (isomer II), respectively, whereas in the other analyzed fruits the amount of this metabolite was similar for all of them. In all species under study, both isomers of (epi)catechin dimers accumulated in higher proportion than (epi)afzelechin isomers. Although the detected concentrations of the latter isomers were small, there were some differences among the fruits of the different *Fragaria* species.

Accumulation of Anthocyanins. Fruit color is one of the most important quality characteristics of cultivated strawberry. It is mostly due to derivatives of the anthocyanidin pelargonidin, the predominant pigment associated with the bright red color, and cyanidin, a minor pigment associated with dark red color.[1] Studies on the composition of anthocyanins have been performed on strawberry and red raspberry cultivars,[26,27] and they have been reported to have antioxidant properties and antiproliferative activity in cancer.[38]

LC-MS analysis revealed clear differences among the fruits of the different *Fragaria* species (Figure 5). In the octoploid species,

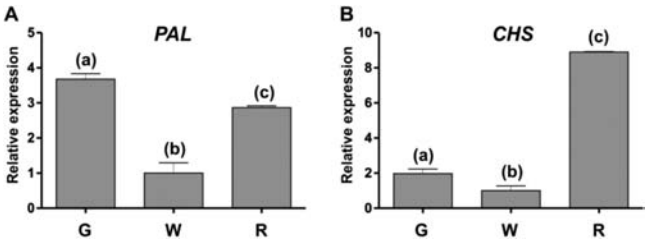

Figure 6. *PAL* (A) and *CHS* (B) expression levels during ripening of strawberry fruits (cv. Camarosa). Values are normalized to the interspacer gene, which was used as control and set to 1. Error bars show the standard deviation (SD) of three independent pools. Different letters indicate a significant difference between samples according to the corresponding ANOVA ($P < 0.05$). G, green fruits; W, white fruits; R, red fruits.

F. chiloensis and *F.* × *ananassa* (cv. Camarosa and cv. Parker), the content of pelargonidin 3-*O*-glucoside was much higher than that of cyanidin 3-*O*-glucoside, whereas in the diploid *F. vesca* their contents were similar. In the hexaploid *F. moschata*, in which the total anthocyanins content was the lowest, the accumulation of cyanidin 3-*O*-glucoside was much higher than that of pelargonidin 3-*O*-glucoside. Finally, the pelargonidin 3-*O*-rutinoside content was minor compared with the other two analyzed anthocyanins, with the greatest accumulation in cv. Camarosa and cv. Parker of *F.* × *ananassa*, whereas in the case of *F. vesca*, *F. moschata*, and *F. chiloensis* this metabolite was almost undetectable.

Expression Analysis of Flavonoid Biosynthesis Genes. In the same ripe fruits of the selected genotypes the mRNA levels corresponding to four genes (*PAL*, *C4H*, *CHS*, and *F3′H*) encoding for key enzymes of the flavonoids pathway were determined by qRT-PCR. It should be noted that expression values by qRT-PCR do not give information on the absolute content of transcripts, but provide only sensitive information on the relative content of transcripts for one gene among the different genotypes. Therefore, comparison among different samples can be performed only gene by gene.

PAL catalyzes the first step in the phenylpropanoids pathway and leads to multiple classes of phenolic products.[9] Throughout strawberry fruit development, the expression of this gene is higher in both green and red fruits compared to white fruits (Figure 6A). This is in agreement with the higher content of stage-specific flavonoids at these two fruit developmental stages.[39] The enzyme CHS catalyzes the stepwise condensation of three acetate units starting from malonyl-CoA with *p*-coumaroyl-CoA to yield 4,2′,4′,6′-tetrahydroxychalcone, which is rapidly isomerized to naringenin, the first stable compound in flavonoid biosynthesis[7] (Figure 1). The expression of the *CHS* gene is highest at the red stage of fruit development (Figure 6B). This is in agreement with the high accumulation of anthocyanins in the red fruits.[39]

The *PAL*, *C4H*, *CHS*, and *F3′H* transcript levels in the ripe fruits of selected *Fragaria* species show a great variability among genotypes (Figure 7). The phenylpropanoid content observed in the analyzed genotypes can be related to *PAL* and *C4H* expression levels (Figure 7A,B). *F. moschata*, *F. chiloensis* and cv. Camarosa of *F.* × *ananassa*, which showed higher values for *PAL* transcript level, displayed elevated levels for total phenolic acid derivatives (Figure 2). Specifically, *F. chiloensis* presented the maximum content of cinnamoyl-D-glucose ester in agreement with higher and lower expression for the *PAL* and *C4H* gene, respectively. On the other hand, cv. Camarosa belonging to

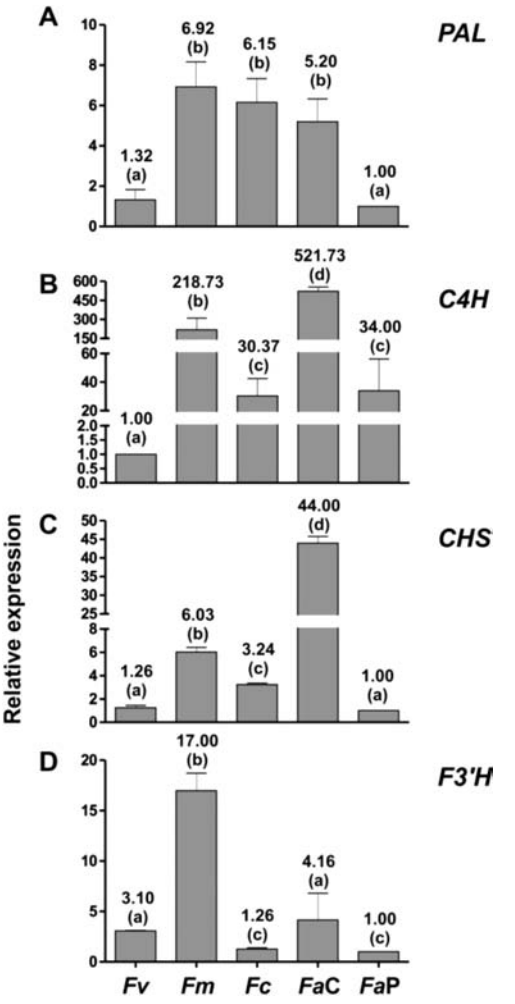

Figure 7. Relative expression profiles of genes involved in flavonoid biosynthesis: (A)*PAL*; (B)*C4H*; (C)*CHS*; (D)*F3′H*. Analysis was performed with primers specific for the five genes. Values are normalized to the interspacer gene, which was used as control and set to 1. Error bars show the standard deviation (SD) of three independent pools. Different letters indicate a significant difference between samples according to the corresponding ANOVA ($P < 0.05$). *Fv*, *F. vesca* (Reina de los Valles); *Fm*, *F. moschata* (Caprón Royal); *Fc*, *F. chiloensis* (Red Chiloensis); *Fa*C, *F.* × *ananassa* (cv. Camarosa); *Fa*P, *F.* × *ananassa* (cv. Parker). Fruits were analyzed at ripe stage.

F. × *ananassa* showed the highest *p*-coumaroyl-D-glucose ester concentration (Figure 2), which seems to be related to the highest expression for the *C4H* gene (Figure 7B).

The maximum *CHS* expression level was observed in cv. Camarosa of *F.* × *ananassa* (Figure 7C), in agreement with high total anthocyanin content observed in this variety (Figure 5). Nevertheless, the wild variety Reina de los Valles of *F. vesca* accumulated a similar total anthocyanin amount (Figure 5) but the *CHS* transcript level was lower than in cv. Camarosa (Figure 7C). On the other hand, the accumulation of different anthocyanins could be related to relative expression levels of the *F3′H* gene. Dihydrokaempferol is a common precursor for pelargonidin and cyanidin anthocyanins (Figure 1). In this respect, it could be assumed that a low *F3′H* transcript level would lead to massive synthesis of pelargonidin-based anthocyanins at the expense of cyanidin-based anthocyanins. Our results show that the

relative number of *F3′H* messages in the octoploids *F. chiloensis* and *F.* × *ananassa* (cv. Camarosa and cv. Parker) was low (Figure 7D), in agreement with the minor cyanidin 3-*O*-glucoside concentration observed[17] (Figure 5). *F. moschata* species showed a high *F3′H* expression level (Figure 7D), in agreement with a high quantity of cyanidin 3-*O*-glucoside relative to the accumulated pelargonidin 3-*O*-glucoside in this genotype (Figure 5). The high relative content of the 3-*O*-glucosides of cyanidin to pelargonidin in *F. vesca* cannot be explained solely with the *F3′H* transcript levels.

Whereas in the case of the phenolic acid derivatives and anthocyanins there is a good relationship among metabolic and transcriptional differences, the observed metabolic differences regarding flavonols and flavan-3-ols accumulation cannot be related to any of the studied genes. Actually, it is still unknown how regulation in the flavonoid biosynthesis pathway occurs. There are several enzymatic steps from the naringenin chalcone to these compounds that have not been afforded in the present study (Figure 1). For that reason it would be necessary to analyze the expression level of other genes encoding enzymes that could act as regulators of this complex metabolic pathway. Moreover, we cannot disregard the possibility that regulation of the metabolic pathway varies among species.[9]

Overall, the LC-MS analysis of different strawberry species revealed clear metabolic differences among the analyzed genotypes. The different levels of phenolic acid derivatives and anthocyanins could be related with *PAL/C4H*, and *F3′H* transcript levels, respectively. Besides, the phenolic composition of strawberry fruits depends on numerous factors such as geographic area, culture techniques, variety, and ripening stage of the fruits, all of them determinants in the organoleptic characteristic and nutritional value of strawberry fruits. Our results can provide a basis for marker-assisted selection of cultivars with high phenolic contents, which will be of interest for the food industry.

■ ASSOCIATED CONTENT

Supporting Information. Figures S1−S5 and Table S1. This material is available free of charge via the Internet at http://pubs.acs.org.

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■ ABBREVIATIONS USED

Fv, *F. vesca*; *Fm*, *F. moschata*; *Fc*, *F. chiloensis*; *Fa*, *F.* × *ananassa*; C, Camarosa; P, Parker; PAL, phenylalanine ammonia-lyase; C4H, cinnamic acid 4-hydroxylase; CHS, chalcone synthase; F3′H, flavonoid 3′-hydroxylase; LC-MS, liquid chromatography−mass spectrometry; qRT-PCR, quantitative real-time PCR.

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