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Two-Phase Flavonoid Formation in Developing Strawberry (*Fragaria* × *ananassa*) Fruit

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Flavonoids are important secondary metabolites in strawberry as they fulfill a wide variety of physiological functions. In addition, they are beneficial for human health. Previous studies have shown for selected enzymes from the flavonoid pathway that flavonoid biosynthesis shows two peaks during fruit development. We provide optimized protocols for the determination of the activities of the key flavonoid enzymes: phenylalanine ammonia lyase, chalcone synthase/chalcone isomerase, flavanone 3-hydroxylase, dihydroflavonol 4-reductase, flavonol synthase, flavonoid 3-*O*-glucosyltransferase, and flavonoid 7-*O*-glucosyltransferase. Using these protocols we were able to demonstrate two distinct activity peaks during fruit ripening at early and late developmental stages for all enzymes with the exception of flavonol synthase. The first activity peak corresponds to the formation of flavanols, while the second peak is clearly related to anthocyanin and flavonol accumulation. The results indicate that flavonoid 3-*O*-glucosyltransferase activity is not essential for redirection from flavanol to anthocyanin formation in strawberry.

KEYWORDS: Strawberry (*Fragaria* × *ananassa*); fruit ripening; fruit color; flavonoid concentrations; flavonoid enzymes; anthocyanins; flavan 3-ols (catechin, epicatechin, and derived proanthocyanidins); flavonols

INTRODUCTION

Strawberry is an important crop in temperate regions such as Central Europe. The attractive fruits are favored for their excellent taste and are health promoting due to their richness in vitamins, minerals, and antioxidative compounds (*1*−*3*). Fruit color is one of the most important quality characteristics for strawberries with respect to human consumption. It is caused by derivatives of the anthocyanidins pelargonidin (predominant pigment, bright red colors, 4′-hydroxylation) and cyanidin (minor pigment, dark red colors, 3′,4′-hydroxylation) (*4*−*8*). Additionally, flavonols are formed (**Figure 1**), which serve as copigments for fruit coloration. In particular, quercetin (3′,4′-hydroxylation) and kaempferol (4′-hydroxylation) derivatives are described in strawberry (*8*−*13*). Interestingly, strawberry fruits produce another prevalent group of flavonoids during early stages of fruit development. In unripe fruits, large amounts of the 3′,4′-hydroxylated flavan 3-ols catechin, epicatechin, and derived proanthocyanidins are accumulated (**Figure 1**) (*14*, *15*). They are beneficial with respect to their antioxidative capacity (*16*−*18*) and presumably contribute to the restriction of plant pathogens such as gray mold (*Botrytis cinerea*), which infects the strawberry flower but is quiescent until fruit ripening is almost completed (*19*−*22*). However, the presence of proanthocyanidins in fruits results in an astringent taste, and catechin may cause undesirable browning due to an enzyme-mediated reaction (*16*).

Previously it has been reported that the activity of phenylalanine ammonia lyase (PAL) shows two peaks during strawberry fruit development (*23*). Similar observations were made in dihydroflavonol 4-reductase (DFR) gene expression studies during fruit ripening (*24*). It was assumed that this could be related to the different functions of the different flavonoid classes formed during early and late fruit development. PAL is a key enzyme located at the interface between primary and secondary metabolism. It catalyzes the conversion of the amino acid phenylalanine into cinnamic acid, which is an important intermediate in the biosynthesis of flavonoids (*25*). However, derivatives of cinnamic acids are the precursors for a broad variety of secondary metabolites (e.g. cinnamic acid derivatives, flavonoids, lignin, coumarins, stilbenes), and the activity of PAL alone therefore provides only limited information with respect to flavonoid formation. To get a more comprehensive insight into the changes occurring in flavonoid metabolism during

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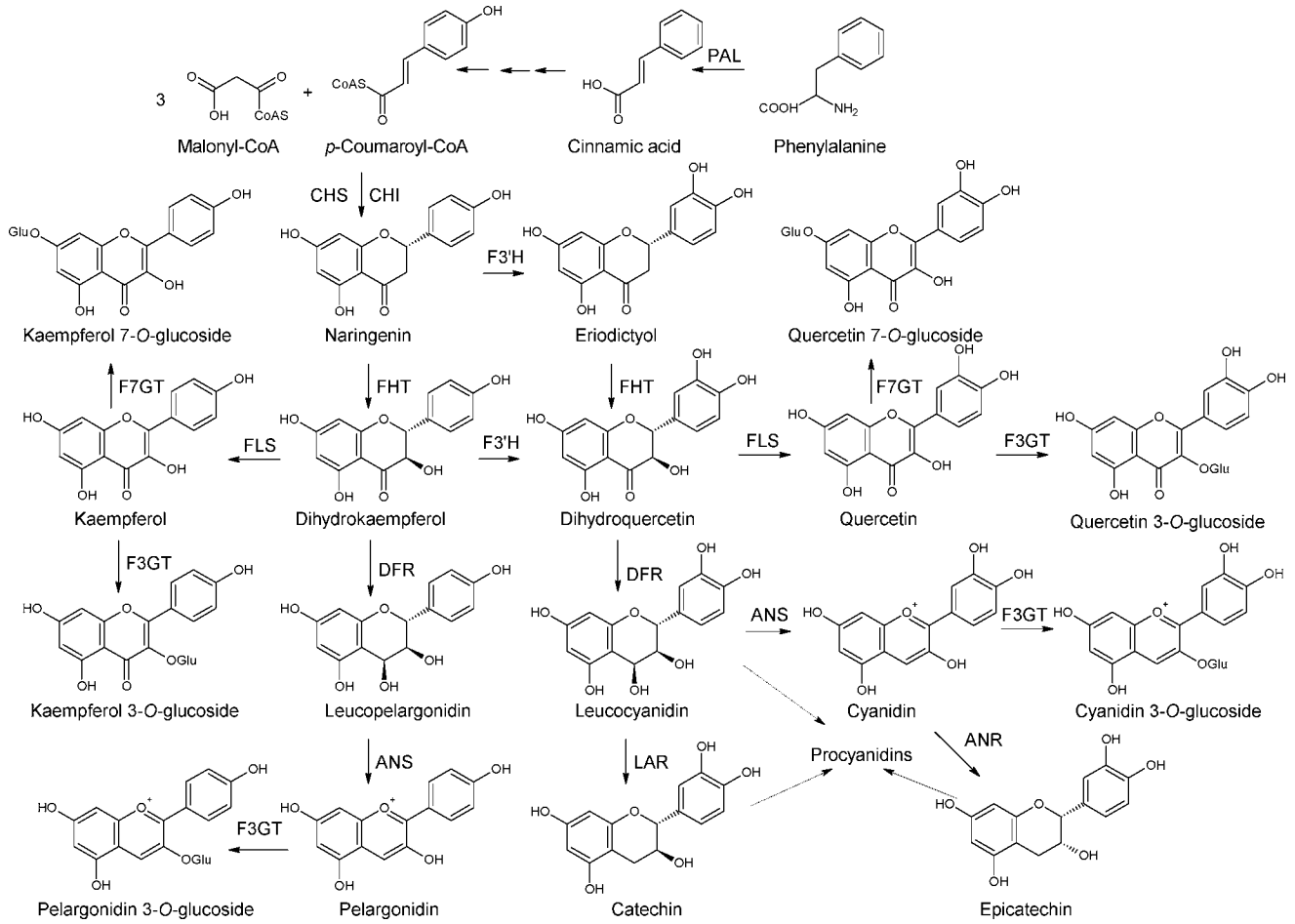

Figure 1. Flavonoid biosynthesis in strawberry fruits.

strawberry fruit development, we investigated six enzymes from the flavonoid pathway for their activities during fruit ripening: PAL, chalcone synthase/chalcone isomerase (CHS/CHI), flavanone 3-hydroxylase (FHT), DFR, flavonol synthase (FLS), flavonoid 7-*O*-glucosyltransferase (F7GT), and flavonoid 3-*O*-glucosyltransferase (F3GT). In addition, the concentrations of different flavonoid classes were determined. In this study we were able to demonstrate that all enzymes with the exception of FLS show two activity peaks during fruit development.

MATERIALS AND METHODS

General Procedure. TLC was performed on Merck precoated cellulose plates (without fluorescence indicator, 1.0571.001, Darmstadt, Germany); radiolabeled substances were detected with a Berthold LB 2842 TLC linear analyzer (Wildbad, Germany). HPLC analysis was performed on a Kontron HPLC system equipped with a 205 gradient former, two T-414 pumps, and a Uvikon UV detector (Eching, Germany). For postcolumn derivatization, a 300 C Gynkothek pump and a Gynkotek VIS detector (Germering, Germany) was used.

Plant Material. The studies were performed on *Fragaria* × *ananassa*, cv. Elsanta. Pooled samples from six developmental stages (25 fruits each) were collected: small-sized (0.7 cm) green fruits (S1), middle-sized (1.5 cm) green fruits (S2), middle-sized (1.5 cm) white fruits (S3), full-sized (2.5 cm) white fruits (S4), turning-stage fruits, 2.5 cm fruit size (S5), and full-ripe red fruits, 2.5 cm fruit size (S6). Fruits were harvested in 2003 and 2004, shock-frozen in liquid nitrogen, and stored at −80 °C. For flavonoid analysis, samples were freeze-dried.

Chemicals. L-[U-^{14}C]Phenylalanine (17 GBq/mmol), [2-^{14}C]-malonyl-coenzyme A (2 GBq/mmol), and uridine diphospho D-[U-^{14}C]-glucose (12.1 GBq/mmol) were obtained from Amersham International (Freiburg, Germany). [^{14}C]-Naringenin (0.8 GBq/mmol), [^{14}C]-dihydrokaempferol (0.8 GBq/mmol), and [^{14}C]-dihydroquercetin (0.8 GBq/mmol) were prepared as recently described (*26*). Quercetin (HPLC grade) was purchased from Extrasynthesis (Genay, France); catechin and epicatechin were purchased from Roth (Karlsruhe, Germany).

Phenylpropanoid Analysis. For extraction of phenolic compounds, lyophilized fruits were ground in a ball mill.

HPLC Analysis. The fine powder was extracted with methanol (100%), containing 6-methoxyflavone as an internal standard, for 30 min in a cooled water bath during sonication. After centrifugation the supernatant was evaporated, and the residue was redissolved in small quantities of methanol and injected for HPLC analysis. The phenolic compounds were separated on a 250 x 4 mm i.d. 3 μm Hypersil ODS column (Shandon, U.S.A.), following a stepwise gradient, using mixtures of solvent A (formic acid, 5% in water) and solvent B (methanol) from 95:5 (v/v) to 10:90 (v/ v) with a flow rate of 0.5 mL per min (*27*). The gradient profile used was as follows: 0−5 min, isocratically, 5% B; 5−15 min, 5−10% B; 15−30 min, isocratically, 10% B; 30−50 min, 10−15% B; 50−70 min, isocratically, 15% B; 70−85 min, 15−20% B; 85−95 min, isocratically, 20% B; 95−110 min, 20−25% B; 110−140 min, 25−30% B; 140−160 min, 30−40% B; 160−175 min, 40−50% B; 175−190 min, 50−90% B. For the quantification of flavan 3-ols, a postcolumn derivatization method was employed (*27*). Flavan 3-ols were identified according to their chromatographic behavior on HPLC and thin-layer chromatography and in comparison with previously isolated standards (*28*). Flavonols were selectively detected at 360 nm and identified by their UV absorbance spectra with a diode array detector. Quantification was performed as follows: Catechin and epicatechin were available as standards, procyanidins were calculated as procyanidin B2, flavonols were calculated as quercetin.

Determination of the Anthocyanin Content. A total of 5 g of the fine powder was shaken with 5 mL of MeOH (containing 1% HCl) for

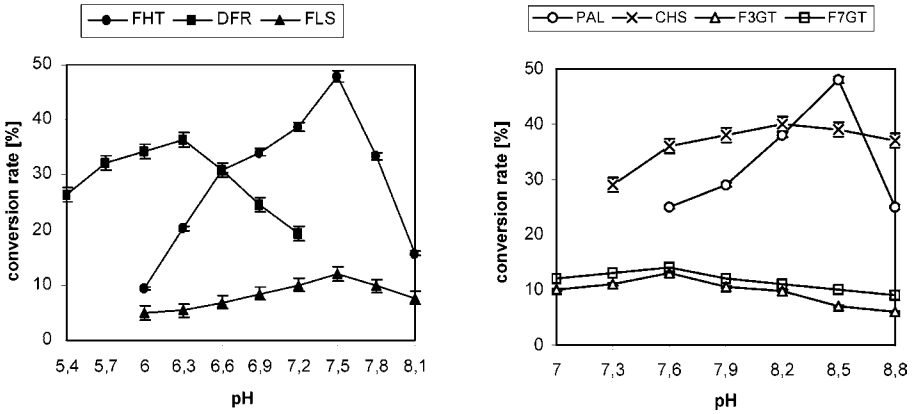

Figure 2. pH-optima of selected enzymes from the flavonoid pathway in strawberry fruits.

30 min. After 5 min centrifugation at 13000*g*, 1 mL of the supernatant was mixed with 1 mL of 4 N HCl and incubated for 60 min at 90 °C. Insoluble residues were removed by centrifugation, the supernatant was diluted 1:15, and absorption at 520 nm was measured. Anthocyanin contents were calculated as mg pelargonidin/g dry weight.

Determination of the Polyphenol Content. Total phenolic content was obtained as a sum of all compounds detected at 280 nm. Not-identified compounds were calculated as gallic acid, flavonols as quercetin, and anthocyanins as pelargonidin.

Determination of Sugar Content. HPLC−sugar analysis was performed on a Dionex BioLC system (Idstein, Germany) equipped with a GP 40 gradient pump, an electrochemical detector ED 40, and a 250 mm × 4 mm analytical Carbo Pac-100 column (Dionex, Idstein, Germany). The sugars were eluated with 1 mL/min 10 mM NaOH within 20 min. For quantification, the sum of the peak areas was calculated as glucose.

Buffers Used. The following buffers were used for the enzyme assays. Buffer 1 (PAL assays): 0.1 M H_3BO_3 + 0.4% Na-ascorbate, pH 8.5. Buffer 2 (CHS assays): 0.1 M Tris/HCl + 0.4% Na-ascorbate, pH 8.25. Buffer 3 (FHT and FLS assays): 0.1 M Tris/HCl + 0.4% Na-ascorbate, pH 7.5. Buffer 4 (DFR assays): 0.1 M KP_i + 0.4% Na-ascorbate, pH 6.3. Buffer 5 (GT assays): 0.1 M KP_i + 0.4% Na-ascorbate, pH 7.5.

Enzyme Preparations. Shock-frozen fruits were ground in a mill. A total of 0.5 g of the fine powder, 0.25 g of quartz sand, 0.25 g of Polyclar AT, and 3 mL of 0.1 M Tris/HCl (containing 0.4% Na-ascorbate, pH 7.25) were homogenized in a mortar. The homogenate was centrifuged for 10 min at 4 °C and 10000*g*. To remove low molecular compounds, 400 μL of the supernatant was passed through a gel chromatography column (Sephadex G25 medium). Protein content was determined by a modified Lowry procedure (*29*) using crystalline BSA as a standard. All data represent an average of at least three independent experiments.

Enzyme Assays. In a final volume of 100 μL: the PAL assay contained 40 μL of enzyme preparation (2.4−8.1 μg total protein), 5 μL of [^{14}C]-phenylalanine (0.063 nmol, 548 Bq), and 55 μL of buffer 1; the CHS/CHI assay contained 40 μL of enzyme preparation (2.4−8.1 μg total protein), 5 μL of [^{14}C]-malonyl-CoA (1.5 nmol, 1300 Bq), 5 μL of *p*-coumaroyl-CoA (1.0 nmol), and 50 μL of buffer 2; the FHT assay contained 0.046 nmol of [^{14}C]-naringenin (108 Bq), 30 μL of enzyme preparation (2.1−6.6 μg total protein), 5 μL of 3.48 mM 2-oxoglutarate, 5 μL of 2.01 mM $FeSO_4$·7 H_2O, and 80 μL of buffer 3; and the FLS assay contained 0.046 nmol of [^{14}C]-dihydrokaempferol (108 Bq), 40 μL of enzyme preparation (2.8−8.8 μg total protein), 5 μL of 3.48 mM 2-oxoglutarate, 5 μL of 2.01 mM $FeSO_4$·7 H_2O, and 50 μL of buffer 3.

In a final volume of 50 μL: the DFR assay contained 0.046 nmol of [^{14}C]-dihydroquercetin (108 Bq), 15 μL of enzyme preparation (1−3.6 μg total protein), 5 μL of 5.21 mM NADPH, and 35 μL of buffer 4; and the GT assay contained 2.5 μL of [^{14}C]-UDPG (0.206 nmol, 2300 Bq), 15 nmol of quercetin (dissolved in 2.5 μL of ethylene glycol monomethyl ether), 25 μL of enzyme preparation (1.8−5.5 μg total protein), and 35 μL of buffer 5.

The assays were incubated for 30 min at 30 °C, with the exception of DFR, which was incubated for 15 min at 30 °C. PAL and CHS/CHI assays were stopped with 200 μL of ethyl acetate and 10 μL of acetic acid, and the amounts of product formed were determined on a scintillation counter. FHT, DFR, and FLS assays were terminated by addition of 70 μL of ethyl acetate and 10 μL of acetic acid. To FLS assays were also added 10 μL of 0.1 mM EDTA before the extraction. The organic phases were transferred to a precoated cellulose plate (Merck, Germany). After developing the TLC plates in chloroform/acetic acid/H_2O (10:9:1, v/v/v), conversion rates were determined with a TLC linear analyzer. GT assays were terminated by addition of 10 μL of acetic acid and 30 μL of methanol. The mixture was chromatographed on Schleicher and Schüll 2043b paper using 30% acetic acid as solvent system. The zones containing the labeled products were cut out, and radioactivity was quantified on a scintillation counter. All products were identified as described using authentic substances (*26*, *32*).

RESULTS AND DISCUSSION

Only a few protocols are available in the literature for the determination of flavonoid enzymes from strawberry fruits (*23*, *30*, *31*). Therefore, the assays previously described for apple leaves (*32*) were adapted to the strawberry fruit tissues. The pH-optima were determined for all enzymes of interest: PAL, CHS/CHI, FHT, DFR, FLS, F3GT, and F7GT (**Figure 2**). Leucoanthocyanidin reductase (LAR), anthocyanidin synthase (ANS), and anthocyanidin reductase (ANR) were not included, since the instability of their substrates hampers reliable comparative studies. All enzyme assays were optimized with respect to temperature, incubation time, and protein concentration (data not shown).

Highest specific activities for CHS/CHI were determined at S1 (**Figure 3**). During fruit development, the activity strongly decreased and reached a minimum at S3−S5. At S6, the activity increased again, but showed rather low activities compared to S1 and S2. In contrast, FLS activity could not be detected in the early stages but reached a distinct maximum at S5. All other enzymes investigated clearly showed two activity peaks at S2 and S6 (**Figure 3**). For DFR and F3GT, the peaks at S2 and S6 showed almost the same size, whereas PAL had a distinct maximum at S2. The two activity peaks observed for most enzymes indicate a two-phase flavonoid biosynthesis during fruit development. The specific activities determined correspond to the high phenolic contents present in the young fruits, in particular to catechin, epicatechin, and proanthocyanidins. The respective time courses are given in **Figure 4**, where the contents are expressed as mg/g dry weight, and **Figure 5**, where the total amount per fruit was calculated, which is a result of fruit growing, product formation, and product turnover. During fruit

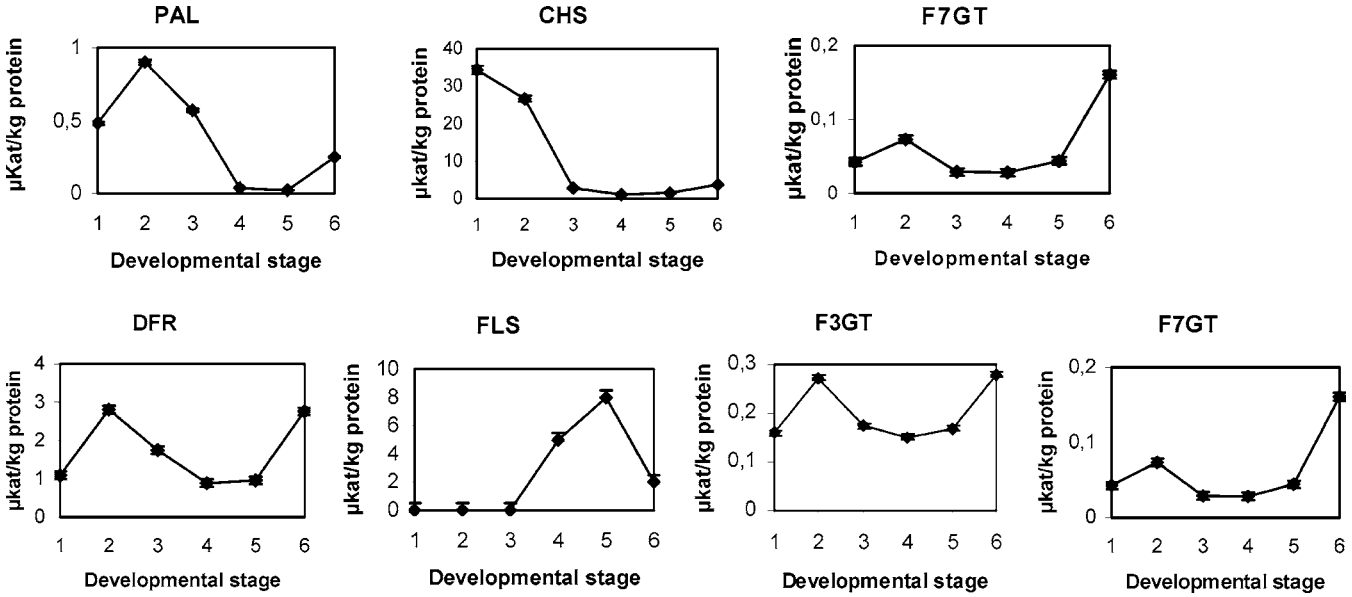

Figure 3. Specific activities [μkat/kg] of selected enzymes from the flavonoid pathway during fruit development.

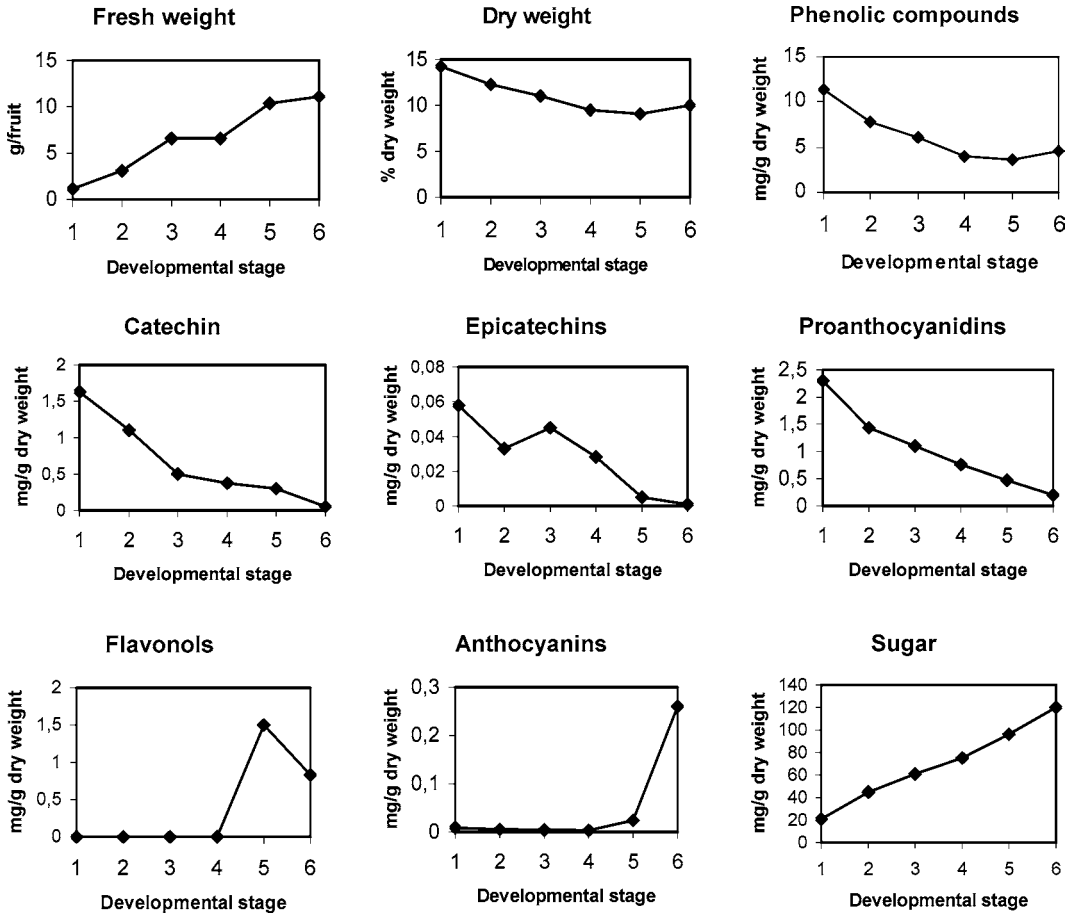

Figure 4. Changes of selected polyphenol and sugar concentrations (in mg/g dry weight) during fruit development.

ripening, the concentrations of flavan 3-ols expressed per mg/g dry weight decreased continuously, while anthocyanins, flavonols, and sugar contents increased simultaneously. Thus, there is an obvious redirection of flavonoid biosynthesis from flavan 3-ol to anthocyanin formation during the complex developmental process of fruit ripening. Since the specific enzyme activities, which are based on the protein content, also consider the increase of the fruit weight during fruit development, the temporal correlation of enzyme activities and product accumulation is reflected by comparison of the time courses in **Figures 3** and **5** rather than in **Figures 3** and **4**.

The different polyphenol classes formed during early and late fruit development seem to fulfill different important functions. In unripe fruits, the presence of astringent flavan 3-ols (**Figure 4**) may contribute to herbivore deterrence along with green color, high acid concentration, and tissue firmness (*33*−*35*). When seeds mature, attraction of harvesting species is attained by the accumulation of the red colored anthocyanins in combination

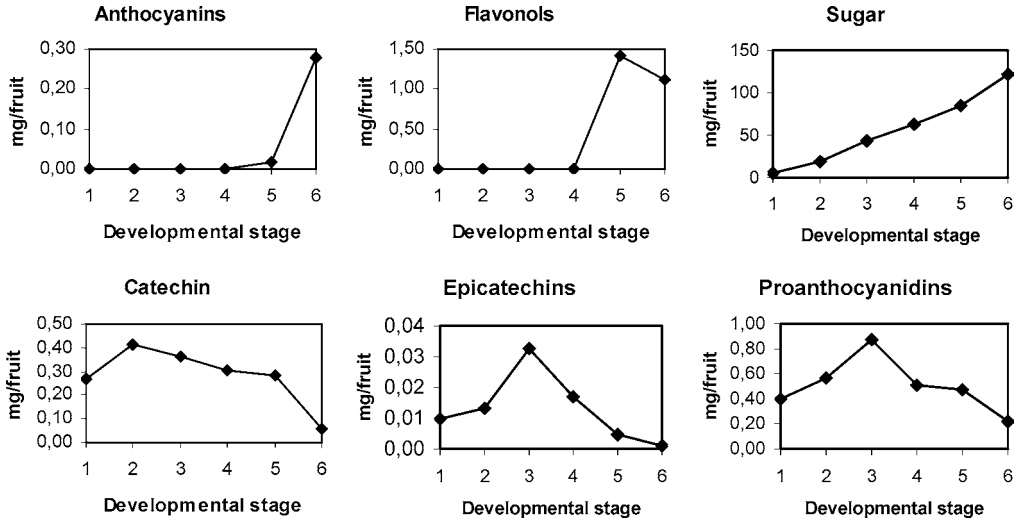

Figure 5. Accumulation of selected polyphenols and sugar during fruit development.

with aroma volatiles, increasing sugar concentrations (**Figure 4**), and a progressive decrease in tissue firmness (*33*−*35*). Formation of flavonols slightly precedes the accumulation of anthocyanins (**Figure 4**), since the uncolored flavonols are important copigments contributing much to fruit and flower coloration by stabilizing the rather unstable anthocyanin chromophore (*33*, *36*).

Interestingly, unripe fruits of strawberry and grapevine do not suffer from gray mold although they may already harbor the pathogen without any visible disease development (*37*−*39*). The involvement of multiple parameters is assumed for the observed pathogen tolerance in the quiescent stage, which is not yet fully understood. The assumption that flavanols may play an important part (*39*, *40*) is supported by our observations of high flavan 3-ol concentrations only in the early developmental stages, which are based on highest specific activities of the related flavonoid enzymes.

There are indications that two-phase flavonoid formation during fruit development may occur also in other crops. For raspberry it could be shown that two different genes encoding PAL are expressed during fruit ripening, both at the earliest and at late developmental stages (*34*). Similar reports are available on grape, where a distinctly reduced expression of seven genes of the anthocyanin pathway was observed between 4 and 8 weeks post-flowering (*41*). However, Waters et al. (*42*) have recently shown a concerted increase in flavonoid gene expression in young grapes but could not observe a similar boost in the late stages during anthocyanin formation. In contrast, expression of genes encoding PAL, CHS, FHT, DFR, and ANS could be observed only in bilberry flowers and in ripening but not in young berries (*43*).

Red grapes (*Vitis vinifera*) represent a more complex system since anthocyanins are formed only in the berry skin (*44*, *45*). The presence or absence of F3GT seems to be a key factor for the formation of red or white grapes (*41*). Furthermore, it is assumed that in red grapes mainly the F3GT activity is decisive for the redirection of flavonoid biosynthesis from proanthocyanidin to anthocyanin formation during fruit ripening (*41*, *46*, *47*). The first biosynthetic steps catalyzed by CHS, CHI, FHT, and DFR are common to the formation of both types of flavonoids, but subsequent biosynthetic pathways are most divergent (**Figure 1**). Whereas the synthesis of catechin and epicatechin is performed via the action of LAR or ANS/ANR in the unripe fruit, the colored anthocyanins of the ripening fruit are produced by ANS/ F3GT (**Figure 1**). However, Given et al. (*35*) reported a parallel course of PAL and F3GT in developing strawberry fruits, although in this study enzyme activities were generally low in the early stages. In our studies, F3GT activity in the early stages was comparable to the late stage (**Figure 3**). Thus, F3GT does not seem to be the key factor in strawberry for the redirection of flavonoid biosynthesis during fruit ripening as suggested for grape.

It is obvious that the two-phase flavonoid biosynthesis has to be tightly regulated with respect to the developmental stages of the fruit. It may be assumed that different types of transcription factors are involved in the regulation as was shown for other plants (*48*). The regulation could act on only one gene or a few genes or, in the other extreme case, there could be two completely different sets of genes for all steps of the pathway coding for the respective early and late biosynthetic enzymes. Further work on this topic is in progress.

ABBREVIATIONS USED

CHS/CHI, chalcone synthase/chalcone isomerase; DFR, dihydroflavonol 4-reductase; FHT, flavanone 3-hydroxylase; FLS, flavonol synthase; F3GT, flavonoid 3-*O*-glucosyltransferase; F7GT, flavonoid 7-*O*-glucosyltransferase; GT, glucosyl transferase; PAL, phenylalanine ammonia lyase; UDPG, uridine diphospho glucose.

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