Dihydroflavonol 4-Reductase Genes Encode Enzymes with Contrasting Substrate Specificity and Show Divergent Gene Expression Profiles in *Fragaria* Species

Silvija Miosic¹, Jana Thill¹, Malvina Milosevic¹, Christian Gosch¹, Sabrina Pober¹, Christian Molitor², Shaghef Ejaz³, Annette Rompel², Karl Stich¹, Heidi Halbwirth¹

¹ Vienna University of Technology, Institute of Chemical Engineering, Vienna, Austria, ² Institut für Biophysikalische Chemie, Fakultät für Chemie, Universität Wien, Vienna, Austria, ³ Bahauddin Zakariya University, Department of Horticulture, Multan, Pakistan

Abstract

During fruit ripening, strawberries show distinct changes in the flavonoid classes that accumulate, switching from the formation of flavan 3-ols and flavonols in unripe fruits to the accumulation of anthocyanins in the ripe fruits. In the common garden strawberry (*Fragaria × ananassa*) this is accompanied by a distinct switch in the pattern of hydroxylation demonstrated by the almost exclusive accumulation of pelargonidin based pigments. In *Fragaria vesca* the proportion of anthocyanins showing one (pelargonidin) and two (cyanidin) hydroxyl groups within the B-ring is almost equal. We isolated two dihydroflavonol 4-reductase (DFR) cDNA clones from strawberry fruits, which show 82% sequence similarity. The encoded enzymes revealed a high variability in substrate specificity. One enzyme variant did not accept DHK (with one hydroxyl group present in the B-ring), whereas the other strongly preferred DHK as a substrate. This appears to be an uncharacterized DFR variant with novel substrate specificity. Both DFRs were expressed in the receptacle and the achenes of both *Fragaria* species and the DFR2 expression profile showed a pronounced dependence on fruit development, whereas DFR1 expression remained relatively stable. There were, however, significant differences in their relative rates of expression. The DFR1/DFR2 expression ratio was much higher in the *Fragaria × ananassa* and enzyme preparations from *F. × ananassa* recepcptacles showed higher capability to convert DHK than preparations from *F. vesca*. Anthocyanin concentrations in the *F. × ananassa* cultivar were more than twofold higher and the cyanidin:pelargonidin ratio was only 0.05 compared to 0.51 in the *F. vesca* cultivar. The differences in the fruit colour of the two *Fragaria* species can be explained by the higher expression of DFR1 in *F. × ananassa* as compared to *F. vesca*, a higher enzyme efficiency (*Kₐ versus Kₗ* values) of DFR1 combined with the loss of F3'H activity late in fruit development of *F. × ananassa*.

Introduction

The strawberry is an appealing plant model for studying flavonoid metabolism during fruit development, as there is not only a change in the flavonoid classes but also in their B-ring hydroxylation patterns. These hydroxylation patterns switch from mainly dihydroxylated flavan 3-ols and flavonols in unripe fruits to monohydroxylated anthocyanins in ripe fruits [1,2,3]. In the *F. vesca* the ratio of anthocyanins possessing one (pelargonidin type) and two (cyanidin type) hydroxyl groups in the B-ring is almost equal, whereas pelargonidin type anthocyanins are particularly prevalent in the *F. × ananassa*. This is frequently reflected in fruit colouration (Figure S1 in File S1) [1,4]. The changes in the hydroxylation patterns can be achieved in two ways: either via the downregulation of flavonoid 3'-hydroxylase (*F3'H*) expression in late fruit stages or via the presence of a set of dihydroflavonol 4-reductases (DFR) showing different substrate specificities. Recently we have demonstrated that the differing hydroxylation pattern of anthocyanins in *F. vesca* and *F. × ananassa* is reflected in the *F3'H* expression pattern. In *F. vesca*, *F3'H* was highly expressed during all developmental stages. This contrasted sharply with a decline in the expression of *F3'H* observed in *F. × ananassa* [1], also reported for anthocyanidin reductase and leucoanthocyanidin reductase, genes specifically involved in flavan 3-ol formation [5]. To investigate whether DFR substrate specificity could also contribute to the establishment of strawberry fruit anthocyanin hydroxylation patterns, we studied DFR in two species of *Fragaria*.

DFR (EC 1.1.1.219) is an oxidoreductase which catalyzes the NADPH dependent reduction of the keto group in position 4 of dihydroflavonols to produce flavan 3,4-diols (synonym: leucoanthocyanidins), which are the immediate precursors for the formation of anthocyanidins and flavan 3-ols, the building blocks of condensed tannins [6]. DFR competes with flavonol synthase for dihydroflavonols as common substrates and therefore interferes with flavonol formation [7]. DFR thus has a strong influence on
the formation of at least 3 classes of flavonoids, anthocyanin pigments, flavanols (which provide protection against herbivore, pests and pathogens), and flavonols (which act as sunscreens) [8,9]. In addition, DFR is unique in the flavonoid pathway, because it can exhibit selectivity for the B-ring hydroxylation pattern of flavonoid substrates. While the DFRs of many plants accept dihydroflavonols possessing one (dihydrokaempferol, DHK), two (dihydroquercetin, DHQ) or three (dihydromyricetin, DHM) hydroxyl groups within the B-ring, specific DFRs have been described from a few plant species that do not convert DHK into the corresponding leucoanthocyanidin [10,11]. These species do not produce pelargonidin based pigments and therefore lack an orange-red flower colouration. F3’H deficient lines for those species show a white or pale rosy flower colouration. Due to the absence of dihydroxylated precursors the formation of anthocyanins is not possible. A prominent example is petunia (Petunia hybrida) [10] where the biotechnological introduction of a non-specific DFR from maize bypassed a gap in the pathway of anthocyanin formation within the F3’H deficient petunia line RL01 [12], resulting in an orangered flower colouration (Figure 1, centre). To probe this phenomenon, an artificial DHK specific DFR was created via site-directed mutagenesis of the DFR from Gerbera hybrida with an exchange of an asparagine in position 134 into leucine [10]. This gene is patented for flower colour modification via transgenic approaches.

We report here on the isolation of two DFR cDNA clones from strawberry fruits demonstrating distinct but contrasting substrate specificity. The divergent ratio of expression between these DFRs in two Fragaria species contributes to the establishment of the different anthocyanin patterns within these fruits.

Results and Discussion

Cloning of DFR from Fragaria species

The NCBI database lists eight DFR full-length clones (reference date March 31, 2014) from strawberry all presenting at least 95% sequence similarity with the first isolated DFR from F. xanansassa (Accession AF029685 [13]). To identify further putative DFRs we screened the F. vesca genome [14] for the presence of homologues of the well-known DFR. With this approach, a DFR sequence was found presenting only an 82% sequence identity. The paralogous genes were named DFR1 (high similarity to Accession AF029685) and DFR2 (so far unknown). Specific primers were designed for the two DFR variant and used for the isolation of cDNA clones from early and late developmental stages of fruits of F. xanansassa cv. Elsanta and F. vesca cv. Alexandria and cv. Red Wonder. 2–3 allelic variants of both DFR variants were isolated from each cultivar (GenBank IDs KC894042-KC894055, Table 1, Figure 2).

DFR1 consisted of 1026 bp with an open reading frame (ORF) of 341 deduced amino acids (F. xanansassa) and 999 bp with an ORF of 333 deduced amino acids (F. vesca), respectively. DFR2 of both species consisted of 1050 bp with an ORF of 349 amino acids. DFR1 and DFR2 sequences shared 80–83% amino acid sequence identity. The sequence identity of DFR2 was 97–99% between F. vesca and F. xanansassa and 98–100% between F. vesca cv. Alexandria and cv. Red Wonder. DFR1 showed 96–98% sequence identity between F. xanansassa and F. vesca, and 97–100% between F. vesca cv. Alexandria and cv. Red Wonder.

The phylogenetic relationship between the DFRs of the various Fragaria species was analyzed via a neighbor-joining tree that includes further amino acid sequences of DFRs accessible in the GenBank (Figure 3). In this tree, the DFRs of the Rosaceae family formed a separate cluster. Within this cluster the DFR2s from Fragaria form a group together with the DFRs of rose (Rosa xhybrida, AAX12422), apple (Malus domestica, AAO39816, AAO39817), pear (Pyrus communis AAO39810) and hawthorn (Crataegus monogynae, AAX1649). Fragaria DFR1 and DFR2 are clearly revealed in different clusters with DFR2s more closely related to the other Rosaceous DFRs than to DFR1.

Studies with the recombinant enzymes

The cDNA clones were transferred into a pYES expression vector and heterologously expressed in yeast (Saccharomyces cerevisiae). All recombinant enzymes demonstrated functional activity catalyzing the NADPH-dependent conversion of dihydroflavonols into leucoanthocyanidins. Control reactions with preparations from yeast cells harbouring the empty vector did not show DFR activity. DFR1s and DFR2s significantly differed in their acceptance of dihydroflavonols. The recombinant DFR2s converted DHQ and DHM to leucocyanidin and leucoanthocyanidin, but did not accept DHK as a substrate. Recombinant DFR1 s, in contrast, were selective for DHK (Figure S2 in File S1).

The two identified DFR variants were further characterized using the DFR pair isolated from ripe fruits of F. xanansassa cv. Elsanta. Apart from substrate acceptance, no striking differences were observed in the biochemical or kinetic characteristics of the two recombinant enzymes (Table 2). The highest reaction rates were observed in a weak acidic environment. The kinetic data, particularly the low $K_m$ and the high $K_{cat}/K_m$ value, confirmed the selectivity of DFR1 for DHK. DFR2 had a higher specificity for DHQ than for DHM displaying lower $K_m$ values and higher $K_{cat}/K_m$ values with DHQ as substrate (Table 2). Testing of substrates was performed under optimized conditions for the respective substrate and was within the linear range of the reaction. The selected incubation time and protein concentration ensured that the maximum conversion rate of the best substrate did not exceed 50%. As frequently observed in heterologous expression systems, the substrate selectivity was less obvious, when the protein was present in excess and incubation time was extended beyond the linear range of the reaction. The distinct substrate specificity of the DFRs, however, was confirmed in assays in which DHK and DHQ were simultaneously offered as substrates. When DHK and DHQ were present in equimolar amounts, DFR1 exclusively converted DHK to leucoanthocyanidin whereas in assays with the recombinant DFR2 only the formation of leuocyanidin could be observed (Figure S2 in File S1).

To date, only two variants of DFRs have been reported in the literature: non-specific DFRs, converting all types of dihydroflavonols, and specific DFRs converting only DHQ and DHM. DFR1 from Fragaria species represents a third variant of DFR, which prefers DHK. The simultaneous presence of several DFR genes has been demonstrated in several plant species [15,16,17,18,19]. It is important to note that gene copies may encode enzymes with different substrate specificities. Drawing conclusions on the substrate specificity of a DFR just from the observed flavonoid hydroxylation pattern should therefore be avoided.

Sequence analysis

DFR1 and DFR2 sequences were analyzed for systematic differences that might be deciding factors for the differing substrate acceptance. The translated amino acid sequences demonstrated the highest divergence at both the N- and C-terminus (Figure 2). DFR2 of both Fragaria species had 349 aa and was generally longer than DFR1, which had 341 aa in F. xanansassa and only 333 aa in F. vesca. Apart from the variable termini, the alignment of the sequences from aa 6–330 (numbering according to DFR1)
Figure 1. Simplified flavonoid pathway demonstrating the influence of DFR substrate acceptance on the establishment of flower colour.
doi:10.1371/journal.pone.0112707.g001

Table 1. DFR cDNA clones isolated from Fragaria species.

| DFR variant | Accession No | Species   | cultivar   | stage   |
|-------------|--------------|-----------|------------|---------|
| DFR1        | KC894042     | *F. vesca*| Alexandria | early   |
| DFR1        | KC894043     | *F. vesca*| Alexandria | late    |
| DFR1        | KC894044     | *F. vesca*| Alexandria | late    |
| DFR1        | KC894045     | *F. vesca*| Red Wonder| early   |
| DFR1        | KC894046     | *F. vesca*| Red Wonder| late    |
| DFR1        | KC894047     | *F. ×ananassa*| Elsanta | early   |
| DFR1        | KC894048     | *F. ×ananassa*| Elsanta | late    |
| DFR2        | KC894049     | *F. vesca*| Alexandria| early   |
| DFR2        | KC894050     | *F. vesca*| Alexandria| late    |
| DFR2        | KC894051     | *F. vesca*| Alexandria| late    |
| DFR2        | KC894052     | *F. vesca*| Red Wonder| early   |
| DFR2        | KC894053     | *F. vesca*| Red Wonder| late    |
| DFR2        | KC894054     | *F. ×ananassa*| Elsanta | early   |
| DFR2        | KC894055     | *F. ×ananassa*| Elsanta | late    |

doi:10.1371/journal.pone.0112707.t001
presence of an aspartic acid in the petunia DFR sequence, which contrasts with the more frequently occurring asparagines, was suggested to determine the inability of converting DHK [10]. In addition, the exchange of the asparagine in the gerbera DFR into a non-polar leucine converts the non-specific DFR into a ‘DHK specialist’ [10]. The crystal structure obtained from a recombinant Vitis vinifera DFR confirmed the importance of this position (in this case aa 133). Asparagine 133 coordinates the dihydroflavonol substrate via interaction with the hydroxyl groups in position 3’ and 4’ [20]. In the DFRs from strawberry, amino acid 133 is an asparagine in DFR2 but an alanine in DFR1. The presence of a non-polar amino acid in the DHK-specialist is in line with [10]. The observed substrate specificity of DFR2, however, cannot be explained by the presence of the asparagine, because this is also found in the non-specific DFR from gerbera.

Relevance of DFR for the anthocyanin hydroxylation pattern in Fragaria species

The expression profiles of DFR1 and DFR2 were studied in the strawberry fruits. Botanically they are not berries but aggregate fruits, where the so-called 'seeds' are the real Fragaria fruits (achenes). The edible part which is commonly referred to as the fruit stems from the receptacle. The polyphenol profile and proteome varies between the tissues [21,22,23]. For this reason, receptacles and achenes were studied separately. The quantitative Real-time PCR data for the DFRs were normalized against actin (Figure S3 in File S1) and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (Figure 4). With both housekeeping genes comparable results were obtained with only slight differences for the early stages of the F. xananassa receptacle (Figure 4). In the receptacle of both species, the profile of DFR2 expression fluctuated during fruit development while for DFR1 expression this was less pronounced. In the achenes, the DFR expression increased during fruit ripening in both species. In F. vesca, DFR2 expression was drastically higher than the DFR1 expression in both tissues during all developmental stages and showed two maxima for the receptacle during stage 1 and 3 with a decline in stage 2 (Figure 4). The highest DFR1 expression in the F. vesca fruits was observed in S3 and S4 of the achenes. In F. xananassa, DFR2 expression was higher in late developmental stages of the fruits, whereas DFR1 expression remained near stable along the different stages of fruit development and ripening. In the first three stages, DFR1 expression was higher than DFR2 expression, in the later stages the ratio reversed, but DFR1 expression was still significantly higher than in the F. vesca receptacle (Figure 4): This was reflected by a differing substrate acceptance of enzyme preparations obtained from strawberry fruits (Figure 5). Preparations from F. vesca receptacles demonstrated higher specific activity with DHQ other than with DHK in all the developmental stages of the fruit. Preparations from F. xananassa displayed variable dihydroflavonol preference during fruit development with higher specific activity with DHK as a substrate compared with DHQ in S1-S3, and still lower ones in S4-S6. DHK acceptance of preparations from F. xananassa, however, was always higher than with preparations from F. vesca, even in the late developmental stages (Figure 5). Profiles of gene expression and enzyme activities were not always consistent. This was particularly the case in the achenes obtained from S3 and S4 fruits of F. vesca with drastically lower enzyme activity observed than what could have been expected from the high DFR expression levels. We assume, however, that this could be a problem related to increased levels of polyphenols as well as proteins and lipids in the achenes which might hamper the enzyme activity measurements. In F. annanassa, this discrepancy was observed to a much lower extent, which is in

Figure 2. Multiple alignment of the deduced amino acid sequences encoded by DFRs isolated from F. vesca cv Alexandria during early (KC894042, KC894049) and late (KC894043, KC894044, KC894050, KC894051) stages, cv. Red Wonder early (KC894045, KC894052) and late (KC894046, KC894053) stages and F. xananassa cv. Elsanta early (KC894047, KC894054) and late (KC894048, KC894055) stages. Abbreviations: RW: Red Wonder, Alex: Alexandria, Es: Elsanta, es: early stage, ls: late stage. doi:10.1371/journal.pone.0112707.g002

revealed 36 points of distinct differences. 12 of the differences are located in a region, which was identified by Johnson et al. [10] as being relevant for determining substrate specificity (aa 126–170 in the gerbera DFR corresponding to 128–172 in DFR1). Position 134 in the gerbera DFR is of particular interest in this regard. The
line with the fact that wild species frequently show increased levels of polyphenols in comparison to their domesticated counterparts [1]. To contrast with S1 and S2 receptacles of \textit{F. xananassa} DFR expression was relatively low compared to enzyme activity. Harmonized profiles, however, can not necessarily be expected. The life span of the DFR enzymes in the cell is completely unknown, and observed activity could be a cumulative result of DFRs produced during different stages, possibly resulting in a shift of maxima between stages. It cannot even be excluded that DFR activities in very early stages result partially from DFR expression in the flowering period. In addition to this, post-transcriptional regulation may play a role.

It would appear that DFR does help to determine the pattern of flavonoid hydroxylation in strawberry fruits. In \textit{F. vesca}, a high

---

Figure 3. Neighbour-joining tree of DFR amino acid sequences of \textit{Fragaria} and various species published in the NCBI GenBank. DFR1 \textit{F. vesca} cv Alexandria, early stage (KC894042), late stage 1 (KC894043), late stage 2 (KC894044), cv Red Wonder early stage (KC894045), late stage (KC894046), \textit{F. xananassa} cv. Elsanta, early stage (KC894047), late stage (KC894048); DFR2 \textit{F. vesca} cv Alexandria, early stage (KC894049), late stage 1 (KC894050), late stage 2 (KC894051), cv Red Wonder early stage (KC894052), late stage (KC894053), \textit{F. xananassa} cv. Elsanta, early stage (KC894054), late stage (KC894055), \textit{Angelonia angustifolia} (KF285561), \textit{Camellia sinensis} (AAT84073), \textit{Citrus sinensis} (AAS00611), \textit{Crataegus monogyna} (AAX16491), \textit{Delphinium belladonna} (BAF49325), \textit{Garcinia mangostane} (ACM62744), \textit{Gerbera hybrida} (CA978930), \textit{Malus domestica} (AAO39816, AAO39817), \textit{Medicago truncatula} (AAR27014, AAR27015), \textit{Onobrychis viciifolia} (AEF14420), \textit{Petunia hybrida} (AAF60298), \textit{Pyrus communis} (AAO39818), \textit{Rosa hybrida} (AAX1242), \textit{Triticum aestivum} (AAQ77347), \textit{Vitis vinifera} (AAX12423, XP002275531), \textit{Vaccinium macrocarpon} (AAL89715, AAL8971, AAX12420). The bootstrap values are indicated next to the relevant nodes (1000 replicates).

doi:10.1371/journal.pone.0112707.g003
F3'H expression during all developmental stages continuously promotes the availability of flavonoids with a 3',4'-dihydroxylation pattern including DHQ which is converted into leucocyanidin by the enzyme encoded by the highly expressed DFR2. As leucocyanidin is the immediate precursor to the formation of dark-red cyanidin based pigments, this results in a drastically higher cyanidin:pelargonidin ratio in the F. vesca fruits (0.51) compared to F. 6 ananassa (0.05). Total anthocyanin concentrations were, however, with 125 mg/kg lower than in F. 6 ananassa (350 mg/kg). We assume, however, that in F. vesca an additional factor could be relevant as the observed concentrations of pelargonidin based pigments are higher than would have been expected from the high levels of DFR2 and F3'H transcripts. Measurements of the DFR activity during the four developmental stages of the F. vesca receptacle with DHQ and DHK as substrates confirmed a high DFR activity with a persistent DHQ acceptance in all stages (Figure 5). The relatively high DHK acceptance compared to the low DFR1 expression level is in line with the higher enzyme efficiency compared to DFR2 (Table 2). In addition, it is possible that F3'H activity in the F. vesca fruits is lower than expected from the high transcript levels. However, this could not be verified by enzyme assays as the F3'H is a membrane associated enzyme and is difficult to measure in tissues which are rich in disturbing compounds such as polyphenols, sugars and glucanes [24]. In F. xananassa, the observed transcript levels are in line with the anthocyanin composition of the fruits. Due to the decrease of F3'H transcript levels during fruit ripening, a depletion of 3',4'-hydroxylated dihydroflavonols is expected which prevents

|                        | Recombinant DFR1 (KC894048) | Recombinant DFR2 (KC894055) |
|------------------------|----------------------------|-----------------------------|
| pH optimum             | 6.00±1                     | 6.25±1/5.75±2               |
| Temperature optimum [°C]| 40                         | 40                          |
| Temperature stability [°C]| 30                        | 30                          |
| Time linearity [min]   | 25                         | 20                          |
| Protein linearity [µg] | 20                         | 25                          |
| apparent K_m [µmol/kg*s] | 114±1                      | 3.1±1/11.2±3                |
| apparent K_m [µM]     | 0.40±1                     | 0.40±2/3.3±1                |
| K_m/K_m [µs*kg]       | 28±1                       | 7.3±4.9±3                   |

using 1DHK, 2DHQ, 3DHM as substrates.
doi:10.1371/journal.pone.0112707.t002

Table 2. Characterization of recombinant DFR1 and DFR2 from Fragaria xananassa cv. Elsanta obtained from heterologous expression of DFRs (KC894048, KC894055) in yeast.

Figure 4. Quantitative expression of DFR1 and DFR2 normalized to glyceraldehyde 3-phosphate dehydrogenase in receptacle and achenes of Fragaria fruits during the different stages of fruit development. a: F. xananassa receptacle, b: F. xananassa achenes, c: F. vesca receptacle, d: F. vesca achenes. red: DFR1, grey: DFR2. Data were calculated from three biological replicates with at least two technical replicates for each and with error bars representing the standard deviation.
doi:10.1371/journal.pone.0112707.g004
the formation of large amounts of cyanidin, despite the high transcript levels of DFR2. The relatively high DFR1 expression levels compared to F. vesca and the high enzyme efficiency of DFR1 demonstrated by the high $K_{cat}/K_m$ values, however, provides sufficient amounts of precursors to allow the formation of leucopelargonidin as the immediate precursors for pelargonidin formation.

Conclusions

Our studies identified a novel DFR variant in Fragaria sp., which demonstrates an unusual DHK preference. The two DFR variants show divergent expression profiles in both of the two species but also with regards to the fruit development. It is likely that these expression profiles are due to differences in the transcript regulation of the two genes. The higher expression rate of DFR1 and the higher enzyme efficiency of DFR1 is an important precondition for the accumulation of pelargonidin based pigments in F. ×ananassa together with the absence of F3'H expression in the late stages. In F. vesca, a high DFR2 and F3'H expression account for the increased levels of cyanidin based pigments. The novel DFR pair derived from a single species will be a perfect model for future studies into the molecular background behind DFR substrate specificity.

Materials and Methods

Plant material

The studies were performed on fruits of Fragaria ×ananassa cv. Elsanta harvested at the experimental orchard of the Institute of Horticulture and Viticulture (University of Natural Resources and Applied Life Sciences, Vienna, Austria), and F. vesca cv. Red Wonder and cv. Alexandria grown at JKI Dresden-Pillnitz, Germany. The fruits were shock-frozen in liquid nitrogen and stored at $-80^\circ$C. The samples were identical to those recently used for the studies on F3'H in strawberry [25]. Briefly, the six developmental stages of F. ×ananassa were small-sized (0.7 cm) green fruits (S1), middle-sized (1-1.5 cm) green fruits (S2), middle-sized (2-2.5 cm) white fruits (S3), middle-sized (2-2.5 cm) turning-stage fruits (S4), middle-sized (2-2.5 cm) late turning-stage fruits (S5), and full-ripe red fruits, 4 cm fruit size (S6). The four developmental stages of F. vesca comprised small-sized (0.3 cm) green fruits (S1), middle-sized (0.5 cm) turning-stage fruits (S2), late turning-stage fruits with 0.6 cm fruit size (S3) and full-ripe red fruits, 0.8 cm fruit size (S4) [25]. Due to differences in morphology and fruit development, stages of fruit development between the two species were not defined in a similar way. It is possible, however, to distinguish between unripe fruits (F. vesca S1 and F. ×ananassa S1+2), turning stage fruits (F. vesca S2+3 and F. ×ananassa S4+5) and ripe fruits (F. vesca S4 and F. ×ananassa S6). Receptacle and achenes were separated manually from frozen fruits without defrosting the material. The tissues were analyzed separately for enzyme activity and gene expression.
Chemicals
(2-14C)-Malonyl-coenzyme A (55 mCi/mmol) was purchased from New England Nuclear Corp. GmbH (Vienna, Austria). (14C)-Labeled flavonoids DHK, DHQ, and DHM were synthesized as previously described [26,27] using recombinant F3'H from *Sallya heterophylla* and F3'H from *Tagetes erecta*.

Cloning of DFR cDNAs from Fragaria species
mRNA was isolated from strawberry fruits with μMACS mRNA Isolation Kit (Miltenyi Biotec, Germany). cDNA was prepared using the SuperScript II Reverse Transcriptase (Invitrogen, Carlsbad, CA) and the oligo(dT) anchor primer GAC-CACGCGTATCGATGTCGAC(T)16 V. The genome of *F. vesca* was screened for sequences with high similarity to a DFR from *F. x ananassa* (AY695812) using the tools available at www.rosaceae.org.

Specific primers [DFR1_f: ATGGGGGTGGGAGCTGAAATC, DFR1_r: TCAACCAGCCCTGCCCTTT, DFR2_f: TGTCAAGAAGATAGAAGATAGA] were designed on the basis of the detected sequences. Proof reading PCR was carried out using the Expand High Fidelity Plus PCR System (Roche, Austria) according to the manufacturer’s instructions. PCR products were ligated into the vector pYES2.1/V5-His-TOPO and transformed into *E. coli* strain Top 10 (Invitrogen, Carlsbad, CA) according to the manufacturer’s instructions. Plasmids were isolated with Wizard Plus SV Minipreps DNA Purification System (Promega, Vienna, Austria) and sequenced by a commercial supplier (Microsynth Austria AG, Vienna, Austria).

Gene expression analysis
The expression of the DFRs was quantified by qPCR using a StepOnePlus system (Applied Biosystems, Darmstadt, Germany) and the SybrGreenPCR Master Mix (Applied Biosystems, Vienna, Austria) according to the supplier’s instruction. The expression was carried out in three independent triplicates, and the data was normalized against two control genes, actin and glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*). The relative expression ratio of a target gene was computed applying the equation according to [26]. The efficiency of the PCR-reaction was determined on the basis of standard curves which were obtained by applying different DNA concentrations. The product specificity was confirmed via melt curve analysis and gel electrophoresis. Primers for DFRs were designed for the isolated DFR sequences (Table S1 in File S1).

Sequence and Phylogenetic Analysis
Multiple alignments were undertaken with Clustal Omega [29,30]. The phylogenetic tree was conducted and bootstrapped with MEGA version 5 [31] using the neighbor-joining method and 1000 replicates.

Anthocyanin determination
For the determination of the anthocyanin content, 10 g of shock-frozen petals were pulverized and mixed with 35 ml 2 M methanolic hydrochloric acid. After shaking for 12 hours at 4°C in an overhead rotator, the suspension was centrifuged for 10 minutes at 19200 x g. 10–140 μl of the supernatant was adjusted with 2 M methanolic hydrochloric acid to a final volume of 1000 μl. The absorption at 520 nm was determined on a DU-65 spectrophotometer (Beckman Instruments). The anthocyanin content was calculated as pelargonidin equivalent using a calibration curve obtained with commercially available pelargonidin chloride (Roth, Germany). For acidic hydrolysis of anthocyanins, 20 μl methanolic hydrochloric acid extract were mixed with 180 μl of 4 N HCL and incubated for 60 minutes at 90°C. After cooling for 10 minutes the mixture was centrifuged for 10 minutes at 10000 x g. The supernatant was adjusted to 200 μl with 4 N HCL and aliquots were used for HPLC analysis [32] using a Perkin Elmer Series 200 HPLC system equipped with a Perkin Elmer Series 200 diode array detector and Total Chrom Navigator, version 6.3.1 (Perkin Elmer Inc). The column was a BDS Hypersil C18 HPLC column, 5 μm, 250×4.6 mm (Thermo Scientific).

Heterologous expression and protein preparation
The vectors harbouring the DFR cDNAs were transformed into yeast strain INVSc1 using the Sc. EasyComp Transformation Kit (Invitrogen, Carlsbad, CA). Preparation of the protein fractions was performed using a modified protocol according to Pompon et al. [33]. Briefly, 250 ml of expression culture was grown in YPGE medium (5 g/l glucose, 10 g/l peptone, 10 g/l yeast extract, 30 ml 100% ethanol) at 28°C and 180 rpm for approximately 6 h, until an OD600 of 0.8–1.2 was reached. After addition of 27 ml 20% (w/v) sterile filtered galactose, the culture was incubated at 28°C and 180 rpm for 15 h. Cells were harvested by centrifugation at 4000 x g for 4°C for 10 min, with TEK (50 mM Tris/HCl, 1 mM EDTA, 100 mM potassium chloride, pH 7.4), and redissolved in 2.5 ml icecold buffer TES-B* (50 mM Tris/HCl, 1 mM EDTA, 0.6 M sorbitol, 2 mM dithiothreitol, pH 7.4). Disruption of cell walls was achieved via vigorous shaking with glass beads for 30 s every minute during a period of 20 min. Glass beads were removed by centrifugation at 4000 x g and 4°C for 10 min. The protein preparation was diluted with 2.5 ml icecold buffer TES-B*, shock frozen in liquid nitrogen and stored at −80°C.

Enzyme preparation
Enzyme preparations from strawberry fruits were obtained by using the protocol of Claudot and Drouet [34] with slight modifications as previously described [24]. To remove low molecular weight compounds, crude enzyme preparations were passed through a gel chromatography column (Sephadex G25, GE Healthcare, Freiburg, Germany). Protein content was determined by a modified Lowry procedure [35] using crystalline bovine serum albumin as a standard.

Enzyme assays
Assays for DFR were performed as described earlier [27,36]. Briefly, the reaction contained in a final volume of 50 μl: 1–10 μl enzyme preparation (2.4–24 μg), 0.048 nmol (14C)-dihydroflavonol, 0.25 nmol NADPH, 44–35 μl 0.1 M KH2PO4/K2HPO4 buffer pH 6.3 containing 0.4% Na ascorbate. The amount of enzyme preparation depended on the recombinant enzyme used and was chosen to ensure a maximum conversion rate with the best substrate at 50%.

Enzyme characterization
All data represents an average of at least three independent experiments. Determination of the pH optimum was carried out as described for the standard DFR assay, but using 0.2 M McIlvaine buffers with pH values between 4.5 and 9.0. Optimal temperature was determined by measuring activities at varying temperatures within 0°C and 60°C. Temperature stability was determined by measuring enzyme activities at 25°C after incubation of the reaction mixture without NADPH at varying temperatures. Kinetic data were calculated from Lineweaver-Burk plots using radiolabeled substrates at varying concentrations.
Supporting Information

File S1  File includes Figures S1–S3 and Table S1. Figure S1: Left: Ripe strawberry fruits of F. vesca cv. Red Wonder. Right: F. ×ananassa cv. Elsanta. Due to differing magnification factors used, fruit size does not appear at a comparable scale. Figure S2: Radioscans of TLC on cellulose from incubation of recombinant DFR2 (left) and DFR1 (right) in the presence of NADPH offering:A: (14C)dihydroquercetin, B: (14C)dihydrokaempferol, C and D: (14C)dihydroquercetin and (14C)dihydrokaempferol in equimolar amounts as substrates. Figure S3: Quantitative expression of DFR1 and DFR2 normalized to actin in receptacle and achenes of Fragaria fruits along the different stages of the fruit development.

References

1. Thill J, Miosic S, Gotame TP, Mikulec-Petkovsek M, Gosch C, et al. (2013) Differential expression of flavonoid 3’-hydroxylase during fruit development establishes the different B-ring hydroxylation patterns of flavonoids in Fragaria × ananassa and Fragaria vesca. Plant Physiology and Biochemistry 72: 72–78.

2. Hallbäck H, Puhl I, Haas U, Zeitler C, Treutter D, et al. (2006) Two-phase flavonoid formation in developing strawberry (Fragaria × ananassa) fruit. Journal of Agricultural and Food Chemistry 54: 1479–1485.

3. Aharoni A, O’Connell AP (2002) Gene expression analysis of strawberry achene and receptacle maturation using DNA microarrays. Journal of Experimental Botany 53: 2073–2087.

4. Sondheimer E, Karash CB (1956) The major anthocyanin pigments of the wild strawberry (Fragaria vesca). Nature 178: 649–649.

5. Almeida JR, D’Amico E, Pires A, Carbone F, De Vos C, et al. (2007) Characterization of major enzymes and genes involved in flavonoid and proanthocyanidin biosynthesis during fruit development in strawberry Fragaria × ananassa. Archives of Biochemistry and Biophysics 465: 61–71.

6. Forkmann G, Heller W (1999) Biosynthesis of flavonoids. In: D Barltrop, K Nakashima, O Meth-Cohn, Sankawa U, editors. Comprehensive Natural Products Chemistry: Elsevier Science, Amsterdam. 713–748.

7. Davies KM, Schwimmi KE, Dorelso SC, Manson DG, Lewis DH, et al. (2003) Enhancing anthocyanin production by altering competition for substrate between flavonol synthase and dihydroflavonol 4-reductase. Euphytica 131: 259–268.

8. Harborne JB (1967) Comparative Biochemistry of Flavonoids. London: Academic Press.

9. Harborne JB, Williams CA (2000) Advances in flavonoid research since 1992. Phytochemistry 55: 481–394.

10. Simon ET, Ryynänen T, Shin B, Cheong H, et al. (2001) Alteration of a single amino acid changes the substrate specificity of dihydroflavonol 4-reductase. The Plant Journal 25: 325–333.

11. Johnson ET, Yi H, Shin B, Oh BJ, Cheong H, et al. (1999) Cytosolic hybrid dihydroflavonol 4-reductase does not efficiently reduce dihydrokaempferol to produce orange pelargonidin-type anthocyanins. The Plant Journal 19: 81–85.

12. Meyer P, Heidmann I, Forkmann G, Saeder H (1987) A new fruit-specific putative dihydroflavonol 4-reductase from Ginkgo biloba in Anthocyanin Biosynthetic Pathway. PLoS ONE 8: e72017.

13. Shimada N, Sasaki R, Sato S, Kaneko T, Tabata S, et al. (2005) A genome of woodland strawberry (Fragaria vesca). Nature genetics 43: 109–116.

14. Thill J, Miosic S, Gotame TP, Mikulec-Petkovsek M, Gosch C, et al. (2013) Synthetic substrate specificities of their Dihydroflavonol 4-Reductase. European Journal of Enzymology 272: 51–64.

15. Thill J, Miosic S, Gotame TP, Mikulec-Petkovsek M, Gosch C, et al. (2013) Substrate Specificity of their Dihydroflavonol 4-Reductase. European Journal of Enzymology 272: 51–64.

16. Paltram R, Renate Paltram and Jürgen Greiner for excellent technical assistance. The authors kindly acknowledge Klaus Olbricht (Julius Kühn-Institute - Federal Research Centre for Cultivated Plants, Institute for Breeding Research on Horticultural and Fruit Crops, Dresden, Germany) for providing fruits of cv. Red Wonder. We would also like to say thank you to Renate Paltram and Jurgen Greiner for excellent technical assistance. Special thanks to Luke McLaughlin for critically reading the manuscript.

Author Contributions

Conceived and designed the experiments: HH KS AR. Performed the experiments: SM MM SP CM SE CG. Analyzed the data: CM JT HH CG. Contributed to the writing of the manuscript: HH KS SM.