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Gene characterization, analysis of expression and in vitro synthesis of dihydroflavonol 4-reductase from *Citrus sinensis* (L.) Osbeck

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Abstract

Dihydroflavonol 4-reductase (DFR, EC 1.1.1.219) catalyzes the reduction of dihydroflavonols to leucoanthocyanins, a key "late" step in the biosynthesis of anthocyanins. In this study we showed that a strong reduction in DFR expression occurs in the non-red orange cultivar (Navel and Ovale) compared to that of the red orange (Tarocco) suggesting that the enzyme could be involved in the lack of production of anthocyanins. Therefore, we isolated and compared the cDNAs, the genomic clones, as well as the promoter regions of blood and blond orange *dfrs*. Our data revealed that the cDNA sequences of pigmented and non-pigmented orange DFRs were 100% homologous and contained a 1017 bp open reading frame which encodes a protein of 338 amino acid residues, corresponding to a molecular mass of 38010.76 Da, with a theoretical p*I* of 5.96. Moreover, we found that there were no significant differences in non-coding regions (introns and 5′ upstream region) of *dfr* sequences. Southern blot analysis of genomic DNA indicated that *dfr* was present as a single copy gene in both cultivars. From these findings the low expression level of blond orange *dfr*, which might play a role in the phenotypic change from blood to blond orange, is thought to be the result of a likely mutation in a regulatory gene controlling the expression of *dfr*. In addition, here we reported the successful expression of orange DFR cDNAs leading to an active DFR enzyme which converts dihydroquercetin to leucoanthocyanidin, thus confirming the involvement of the isolated genes in the biosynthesis of anthocyanins. Moreover, as far as we know, this is the first report concerning the in vitro expression of DFR from fruit flesh whose biochemical properties might be very different from those of other plant organ DFRs.

Keywords: *Citrus sinensis*; Rutaceae; Blond and blood oranges; Dihydroflavonol 4-reductase; Promoter region; RT-real time PCR; In vitro protein expression; Anthocyanins; Juice vesicles pigmentation

1. Introduction

Anthocyanin pigments produce a range of colors in leaves, flowers, seeds and fruits. The biological functions of anthocyanins are diverse and include attracting insects and birds for pollination and seed dispersal, and protection against photo-oxidative damage (Field et al., 2001, Winkel-Shirley, 2001a). The anthocyanin biosynthesis pathway has been extensively studied in a number of species including maize, snapdragon and petunia, and most of the structural genes encoding the enzymes responsible for each step have been isolated from different sources (Holton and Cornish, 1995). The first specific enzyme of the anthocyanin biosynthetic pathway is chalcone synthase (CHS), which condenses malonyl-CoA and 4-coumaroyl-CoA to form tetrahydroxy chalcone (Fig. 1) (Holton and Cornish, 1995). This last compound is isomerized by chalcone isomerase (CHI) to the flavanone naringenin, which is subsequently converted to dihydrokaempferol by flavanone 3′-hydroxylase. Then, the

Abbreviations: FAM, 6-carboxyfluorescein; GSP, gene specific primer; RACE, rapid amplification of cDNA ends; TAMRA, tetramethylrhodamin.

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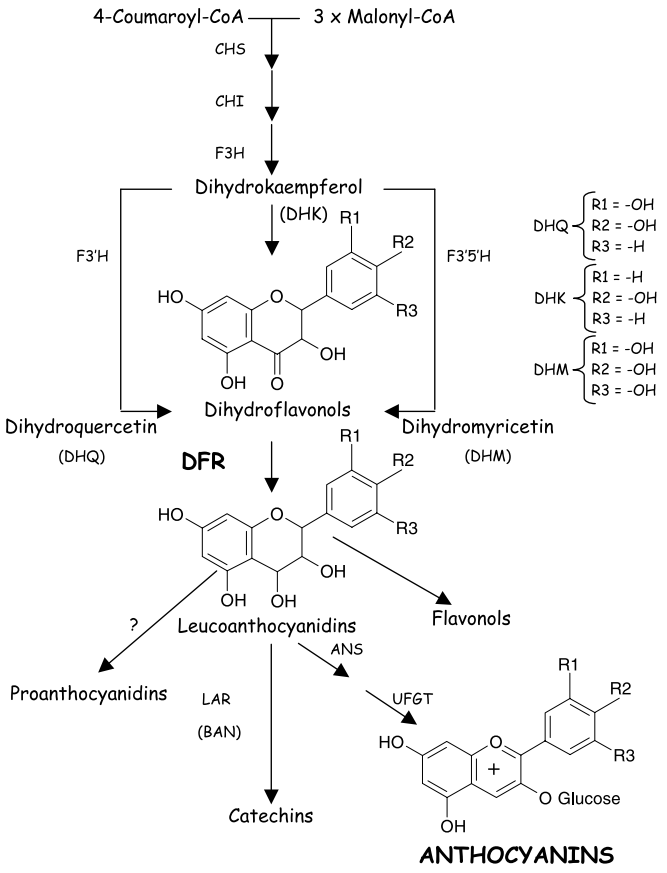

Fig. 1. Outline of the biosynthetic pathway leading to the synthesis of anthocyanins. In the scheme is emphasized the key role of DFR in the production of pigmented anthocyanins and non-pigmented flavonols. Enzyme names are abbreviated as follows: chalcone synthase (CHS), chalcone isomerase (CHI), flavanone 3′-hydroxylase (F3H), flavonoid 3′-hydroxylase (F3′H), flavonoid 3′,5′-hydroxylase (F3′,5′H), dihydroflavonol 4-reductase (DFR), anthocyanidin synthase (ANS), UDP-glucose flavonoid glucosyl transferase (UFGT), flavonol synthase (FLS), leucoanthocyanidin reductase (LAR). "?" represents the unidentified condensing enzyme proposed to produce condensed tannin.

dihydroflavonols, dihydroquercetin and dihydromyricetin, are synthesized from dihydrokaempferol by hydroxylation reactions catalyzed respectively by flavonoid 3′-hydroxylase (F3′H) or flavonoid 3′,5′-hydroxylase (F3′,5′H). Finally, dihydroflavonol 4-reductase (DFR), anthocyanidin synthase (ANS) and UDP-glucose flavonoid glucosyl transferase (UFGT), catalyze the consecutive reactions leading to anthocyanin 3-*O*-glycosides from dihydroflavonols (Fig. 1). The activity of the anthocyanin biosynthetic genes is largely regulated at the transcriptional level and, consequently, the pattern of pigmentation must be specified by the expression patterns of the regulatory genes (Quattrocchio et al., 1993). Mutants in which anthocyanin synthesis is reduced or completely blocked have been identified in numerous plant species (Holton and Cornish, 1995; Quattrocchio et al., 1993). In many cases, these mutations affect enzymes in the flavonoid biosynthesis pathway, but some of them affect regulatory loci. Molecular analysis of these regulatory loci in maize showed that they belong to two gene families encoding transcription factors: the *c*1 family (containing a MYB domain), and the *r* family (containing a basic helix-loop-helix motif). The *c*1 and *r* family activate the entire set of biosynthetic genes, from chalcone synthase to UDP-glucose:flavonoid-3-*O*-glucosyltransferase, with the result that their expression directly determines the pigmentation pattern in maize (Martin and Gerats, 1993). All the regulatory genes identified in other species, such as petunia, control the anthocyanin-specific structural genes but, despite encoding similar proteins, they differ in their activity over the genes common to the synthesis of the multiple classes of flavonoids. In petunia, in fact, the control start point is further on in the pathway operating at the DFR level (Quattrocchio et al., 1993). Moreover, expression assay experiments in which the MYB and bHLH-type regulatory genes have been interchanged between maize and petunia showed that the *chs* gene of maize responds to a combination of petunia regulatory genes, whereas petunia *chs* gene does not (Quattrocchio et al., 1993). This finding indicates that the regulation mechanism could be related to the lack of some *cis*-acting elements in the promoter of the downstream genes rather than to putative modifications in the regulatory protein (Quattrocchio et al., 1993). Other mechanisms of gene-silencing that inactivate genes at the transcriptional level were also identified in the anthocyanin biosynthetic pathway. A transposable element of 6.4 kb was found within the DFR gene of Japanese morning glory. Disruption of the DFR structural gene caused formation of acyanic flowers (Inagaki et al., 1994). Fine control of gene expression and of the encoded enzyme activities is particularly important at branch points in the flavonoid pathway, where the flow of substrates into different flavonoid types is determined. The dihydroflavonols represent one of these branch points since they are the substrates for the production of both pigmented anthocyanins and non-pigmented flavonols, catechins and proanthocyanidins (Fig. 1). Consequently, DFR (EC 1.1.1.219) has to be considered a key enzyme since it catalyzes the NADPH-mediated reduction of dihydroflavonols to leucoanthocyanidins, the immediate precursors of anthocyanidins. Owing to the crucial role of this enzyme in anthocyanin production, DFR genes have been isolated from several higher plants (Belt et al., 1989; Helariutta et al., 1993; Sparvoli et al., 1994; Fischer et al., 2003) and their regulation has been extensively studied in maize (Holton and Cornish, 1995), petunia (Quattrocchio et al., 1993), snapdragon (Holton and Cornish, 1995), and, more recently, in *Arabidopsis* (Winkel-Shirley, 2001b). As regards tree fruits, the regulation of *dfr* expression was carefully investigated in apple (*Malus sylvestris* L. Mill. var. *domestica*) and grape (*Vitis vinifera* L.) skin (Honda et al., 2002; Boss et al., 1996). Northern analysis revealed in both cases that in non-red cultivars the levels of *dfr* expression were lower than in red cultivar (Honda et al., 2002; Boss et al., 1996). In contrast with these results, in some white grape cultivars, such as Riesling, Semillon and Chardonnay, the content of *dfr* transcripts was only slightly lower than the one shown by the red-skinned grapes (Boss et al., 1996), suggesting a more complicated regulation of the anthocyanin pathway. Therefore, a deeper

understanding of the regulation of the expression of DFR gene would provide useful information about the role of this enzyme in relation to the induction of the anthocyanin biosynthetic pathway in tree fruits. Several selected sweet orange [*Citrus sinensis* (L.) Osbeck] cultivars, such as Tarocco, Moro and Sanguinello (blood cultivars) are characterized by the presence, both in the flesh and in the rind, of anthocyanins not usually found in *Citrus* genus and, more specifically, absent in blond orange cultivars such as Navel, Ovale, and Valencia (Rapisarda and Giuffrida, 1992). The presence of the red water-soluble anthocyanins in the edible portion of orange fruits is of special interest because recent studies have described their beneficial effects on health, since they are thought to protect against certain tumors, cardiovascular diseases and aging (Amorini et al., 2003). We previously isolated partial cDNA clones of six enzymes active in the late steps of the anthocyanin biosynthetic pathway (CHS, CHI, F3′H, DFR, ANS and UFGT) from *Citrus sinensis* L. Osbeck (Lo Piero et al., 2001), and showed by non-quantitative RT-PCR that transcripts encoding the "late" enzymes such as DFR, ANS and UFGT are significantly reduced in non-red oranges compared to red cultivars (Lo Piero et al., 2005). In this paper, we present results obtained on the different expression pattern of the DFR gene in the juice vesicles of both blood and blond oranges monitored by quantitative real time RT-PCR. Moreover, to obtain further information on this gene, we have isolated *dfr* genomic homologues as well as the promoter regions from blond and blood orange cultivars to find possible sequence modifications that could explain the different expression in the two cultivars. Finally, an active DFR from blood orange cDNA has been expressed in a cell-free system, this being the first report of an in vitro expression of DFR from fruit tissues. This last result will help to study the kinetic and structural properties of fruit DFR.

2. Results

2.1. Sequence analysis of dfr DNA and cDNA clones and the encoded proteins

The genomic and the cDNA *dfr* clones were isolated from both pigmented (Tarocco) and non-pigmented (Navel) orange juice vesicles as described in Section 4. The cDNA nucleotide sequences of pigmented and non-pigmented orange DFRs were 100% homologous (Fig. 2) and contained a 1017 bp open reading frame which encodes a protein of 338 amino acid residues, corresponding to a molecular mass of 38010.76 Da, with a theoretical p*I* of 5.96. Sequence analysis and comparison with sequences in the databank revealed high identity of the deduced amino acid sequences with higher plant DFR previously isolated (Fig. 2). The higher values for identity and similarity were obtained with DFRs from *Vitis vinifera* (80% and 91%), *Populus tremuloides* (78% and 91%), *Malus domestica* (77% and 92%), *Pyrus communis* (76% and 92%), *Rosa hybrida* (75% and 88%) and *Medicago truncula* (74% and 86%). An N-terminal NADP-binding domain, previously proposed as the dinucleotide binding domain of NADP(H) and NADH-dependent reductases and dehydrogenases (Lacombe et al., 1997), is also present in putative DFR protein of sweet orange. The region predicted to be related to substrate specificity (Johnson et al., 2001) revealed low homology between all DFRs. By comparing of the genomic sequence with that of cDNA, six exons of 118, 170, 195, 160, 193 and 181 bp were identified, separated by five introns of 98, 294, 111, 136, 78 bp, respectively (Fig. 3). The splice junctions with GT dinucleotide in the 5′ donor junction sequence and an AG dinucleotide in the 3′ donor junction sequence of the introns follow Chambon's rule (Breathnach and Chambon, 1981). As with most other eukaryotic genes, the exons were found to have a lower A + T percentage (46.6–60.8% with an average of 54.9%) than the introns (63.1–70% with an average of 66.5%). The clones have a 3′ untranslated region of 162 bp, including the stop codon, determined by comparing the 3′RACE sequence with that obtained with "genome walker" experiments. A plant consensus polyadenylation, AAATTAA, is also found in this region at an upstream distance of the cleavage site in the range of the consensus distance (10–30 nucleotides) for eukaryotic genes (see Fig. 3). The genomic sequences of *dfrs* from pigmented and non-pigmented orange cultivars were highly homologous to each other as only two mutations were found, both of which were located in a non-coding region: an extra T at position + 466 (intron II) and a G instead of an A at position + 1240 (intron IV) were found in the Tarocco *dfr* (Fig. 3).

2.2. Analysis of the dfr promoter region

The 5′ upstream region of both pigmented and non-pigmented oranges *dfr* were isolated by the "genome walker" technique, as described in Section 4, and sequenced. The promoter sequences of *dfr* obtained from pigmented (Tarocco) and non-pigmented oranges (Navel) shared a very high percentage of homology (data not shown) as only one difference at position −931 bp was found: a T was in *dfr* promoter region from pigmented oranges (Tarocco) instead of a C, present in the non-pigmented oranges (Navel). As shown in Fig. 4, a putative transcription start, at −79, was inferred by comparing *dfr* promoters with a consensus sequence for plant gene transcription starts (C/TTCATCA) (Joshi, 1987). Consistent with this, a TATA box upstream of the putative transcription start was also found (Joshi, 1987). Examination of the *dfr* promoter sequence revealed a number of motifs which may be involved in the transcriptional activation of the DFR genes: a putative Unit I, which includes the homologous binding site for the G-box factor and the downstream MYB homologous binding site (Grotewold et al., 1994), was found. Unit I was initially identified in the *chs* promoter and shown to be involved in light induction of the gene; it was shown to be necessary and sufficient to confer photoregulation through the phytochromes and the UV-A/

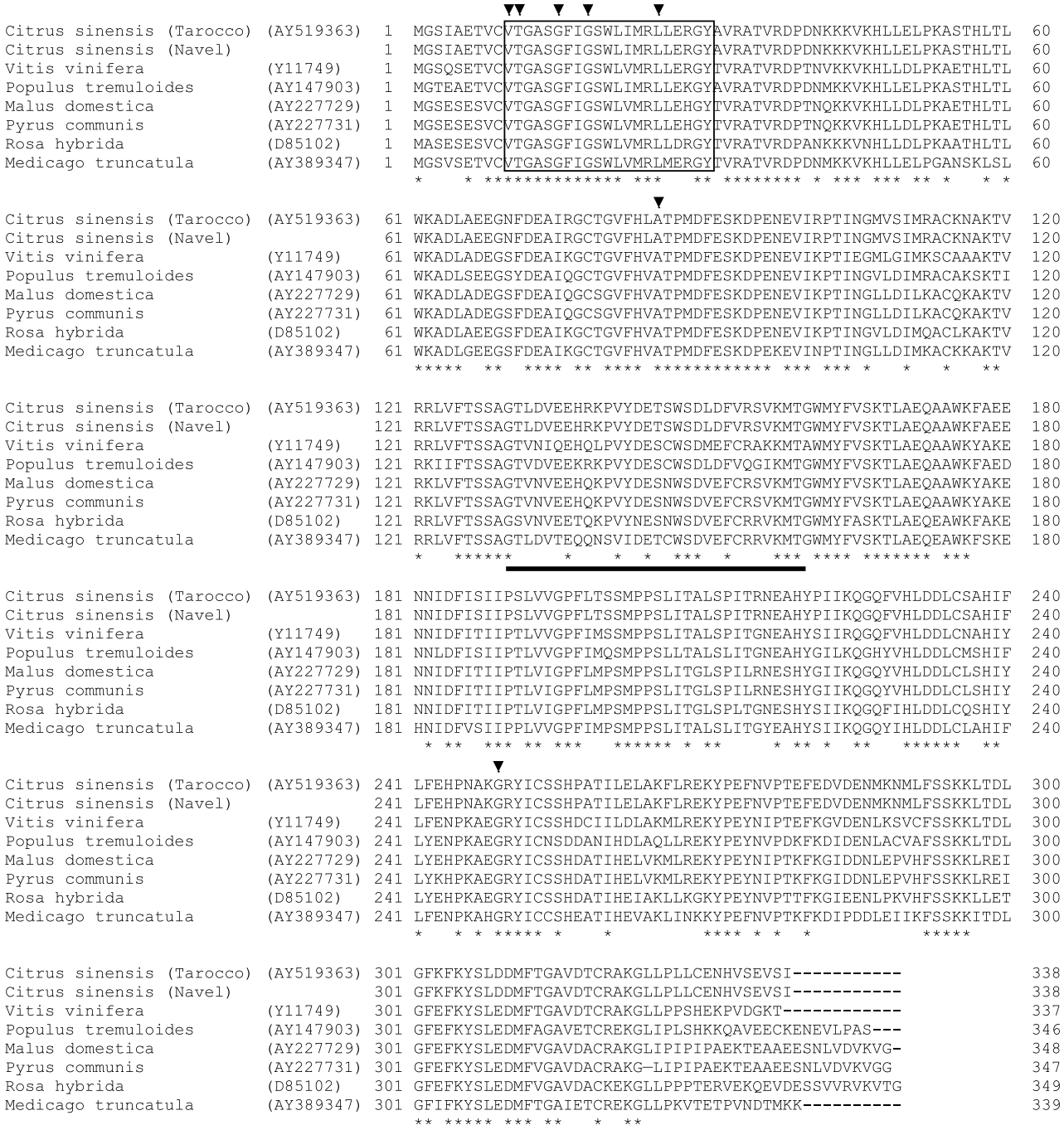

Fig. 2. Alignment of the deduced amino acid sequences of *Citrus sinensis* (Tarocco and Navel) DFRs. Asterisks indicate identical amino acids in all sequences. The boxed region represents a putative NADPH binding domain. The amino acid residues strictly conserved in the mammalian 3β-hydroxysteroid dehydrogenase/DFR superfamily (Lacombe et al., 1997) are marked with black arrow heads. The region predicted to be related to substrate specificity is underlined (Johnson et al., 2001).

blue and UV-B photoreceptor (Gollop et al., 2002). Two putative GA-myb (gibberellic acid activated myb) homologous binding sites are also located upstream of Unit I (Fig. 4). Several core elements for Dof3 (one zinc finger), which have been reported to be involved in tissue specificity and light regulation (Yanagisawa and Schmidt, 1999), were observed in the *dfr* promoters. A sequence with homology to the TACpyAT box of the *chs* promoter (van der Meer et al., 1992), which is responsible for organ-specific expression, was also identified immediately upstream of the TATA box. Moreover, three homologous SBF-1 transcription factor binding sites were also detected in the *dfr* promoter (Lawton et al., 1991).

2.3. Southern blot analysis

DFR gene copy number was assessed by Southern blot analyses using the *dfr* cDNA as hybridization probe.

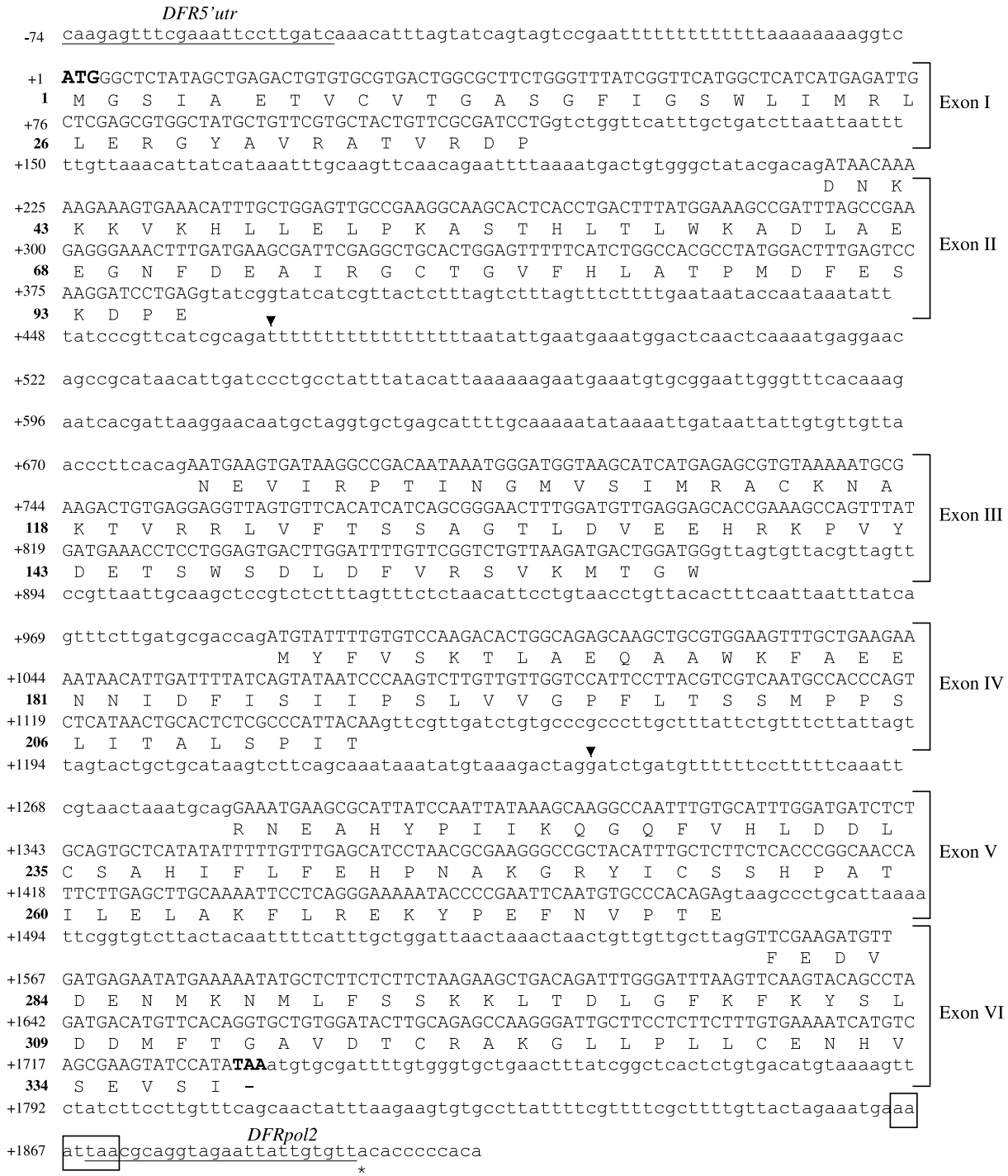

Fig. 3. Nucleotide sequence of the gene encoding DFR from *Citrus sinensis* (Tarocco) with its deduced amino acid sequence. Sequence mutations between blood and blond oranges are indicated by black arrow heads: a T was missed at position +466 and an A instead of a G at position +1240 was in the Navel *dfr*. In the nucleotide sequence, uppercase letters indicate exon sequence and lowercase letters represent introns. The boxed region represents a putative polyadenylation signal AAATTAA as revealed by computer analysis. The asterisk indicates the end of the 3′ untranslated region determined by comparing sequences obtained with 3′RACE and "genome walker" experiments. In bold are indicated the translation start (ATG) and the stop (TAA) signals. The primers used to isolate the genomic clones of *dfrs* are underlined.

Genomic DNAs isolated from mature leaves of Navel and Tarocco oranges trees were digested with EcoRV which does not cut within the DFR genomic clone, EcoRI and AvaII, both of which cut once within the DFR genomic clone, and BstNI that cuts twice within the DFR DNA. As shown in Fig. 5, an identical, simple pattern of DFR hybridizing sequences was obtained from both pigmented and non-pigmented cultivars. Only one band of size greater than 12 kb was detected when the genomic DNAs were digested with *Eco*RV, two bands were found for both

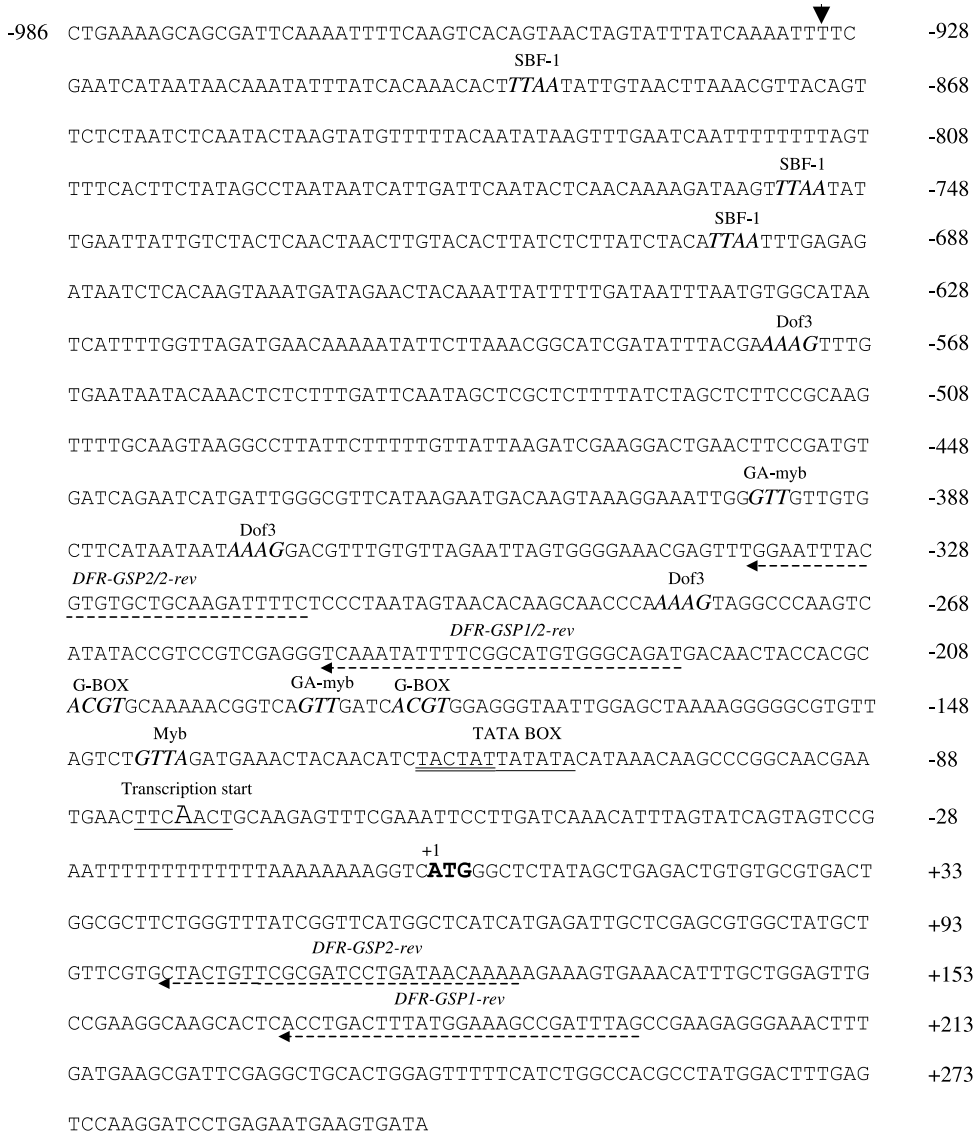

Fig. 4. Analysis of the *dfr* promoter isolated from blood orange (Tarocco) juice vesicles. The *dfr* sequence upstream, the ATG start codon (−986 bp) and 300 bp of the protein coding region are listed. The putative transcription start (uppercase A), the TATA-box (underlined) and the ATG codon (+1, in bold) are indicated. A TACpyAT box overlapping with the TATA-box is twice underlined. Regulatory motifs are indicated above the sequence and the cores are shown in italic. Primers used in the ''genome walker'' experiments are also indicated above the sequence and underlined with broken lines. Sequence mutation between red and non-red oranges is indicated by a black arrow above the sequence: a C instead of a T is present in Navel *dfr* promoter at that position (−931).

*Eco*RI and *Ava*II digests, whereas three bands were obtained when *Bst*NI was used. Taken together, these results suggested that the DFR sequence is present as a single copy gene in the *Citrus sinensis* genome as already found for *H. vulgare*, *A. thaliana* and *Lycopersicon esculentum* DNAs (Kristiansen and Rohde, 1991; Shirley et al., 1992; Bongue-Bartelman et al., 1994).

2.4. Analysis of *dfr* expression in red and non-red orange fruits

The expression profile of *dfr* was investigated in blood and blond oranges using real time RT-PCR. The results are shown in Table 1, which reports the relative transcript levels of *dfr* standardized to the constitutive EF-1α gene expression level and normalized to the Navel $2^{-\Delta\Delta C_T}$. The expression of Tarocco oranges *dfr* was more than 250 times higher than the one measured in the non-pigmented Navel and Ovale orange cultivars, thus suggesting that the phenotypic change from blood to blond might be strictly correlated to the impaired expression of this enzyme. It is worthwhile noting that EF-1α partial cDNA clone was amplified every time in all the studied cultivar types with no significant difference in the C_T values, thus demonstrating that the mRNA samples were intact, and that the strong decrease of *dfr* expression we observed in non-red orange cultivars did not reflect a general decline in mRNA production.

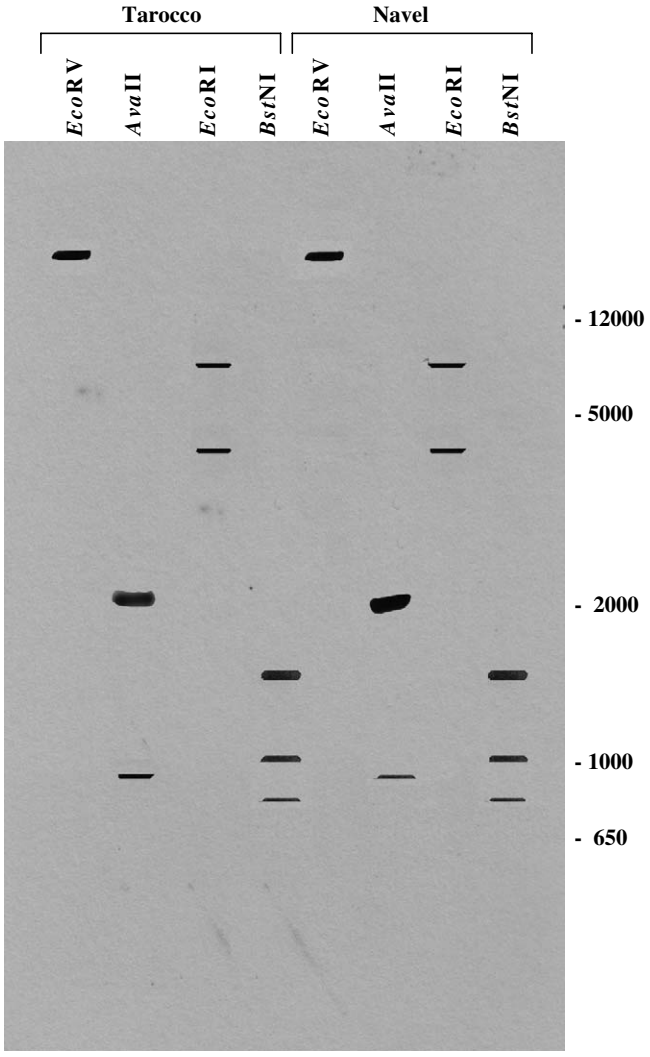

Fig. 5. Southern-blot analysis of *Citrus sinensis* (Tarocco and Navel) genomic DNAs. DNAs were digested overnight with 5 U/ μg DNA of the following restriction enzymes: *Eco*RV, *Ava*II, *Eco*RI and *Bst*NI. Samples were concentrated by sodium-acetate-ethanol precipitation, separated by electrophoresis on 0.7% agarose gel and subjected to Southern-blot analysis. A 1017 bp fragment comprising the entire orange DFR cDNA was radioactively labeled and used as hybridization probe under stringent conditions.

2.5. Measurement of DFR activity in the crude extract of orange fruit

The dihydroflavonol 4-reductase activity was measured both in the pigmented (Tarocco) and non-pigmented (Navel and Ovale) orange fruits, as described in Section 4. As shown in Table 2, enzyme activity was strongly dependent on the type of cultivars; only the red Tarocco cultivar showed detectable DFR activity, whereas both the non-red Navel and Ovale cultivars tested did not.

Table 2
DFR activity of both pigmented (Tarocco) and non-pigmented (Navel and Ovale) orange fruits

Cultivar	DFR specific activity (pkat/mg)
Tarocco	0.2 ± 0.009
Navel	n.d.
Ovale	n.d.

Enzyme activity was measured on crude extracts of orange fruits as described in Section 4. n.d. means not detectable.

2.6. Determination of the functional identity of the cloned dfr cDNAs

To determine the functional identity of the cloned genes, the DFR cDNA was subcloned into pEXP1-DEST, resulting in the construct DFR-pEXP1-DEST which was transcribed and translated in vitro. Subsequently, aliquots of the enzyme extract (10–30 μg) were incubated with dihydroquercetin in the presence of both NADPH and a NADPH-regenerating system. Then, leucoanthocyanidin (non-pigmented) was converted to anthocyanidin (pigmented). Anthocyanidin was quantified as described in Section 4, and the enzyme activity, expressed as the amount of cyanidin formed per second, was estimated to be 0.05 nkat. As expected, negative control samples carried out with extracts containing empty plasmid as DNA template did not show any activity. This finding indicated that recombinant *Citrus* DFR enzymes (Tarocco and Navel) are able to catalyze the conversion of dihydroquercetin to leucoanthocyanidin, a crucial step in the pathway leading to cyanidin 3-*O*-glucoside.

3. Discussion

Cyanidin-3-*O*-glucoside is the major anthocyanin of blood oranges representing 97% of the total pigment content (Maccarone et al., 1983); this gives orange fruits their distinctive antioxidant properties due to the very negative redox potential of cyanidin-3-*O*-glucoside ($E_{1/2} = -405$ mV) (Amorini et al., 2003). Factors such as variety and environ-

Table 1
Analysis of the expression of *dfr* in different cultivars of sweeet orange (*Citrus sinensis* L.) by RT-real time PCR

Cultivar	C_T DFR	C_T EF	ΔC_T	$\Delta\Delta C_T$	Normalized *dfr* amount relative to Navel $2^{-\Delta\Delta CT}$
Tarocco	31.76 ± 0.026	22.68 ± 0.020	9.08 ± 0.044	-8.02 ± 0.079	259.8 ± 14.5
Navel	39.81 ± 0.026	22.71 ± 0.026	17.1 ± 0.040	0	1
Ovale	39.20 ± 0.017	22.75 ± 0.010	16.45 ± 0.026	-0.65 ± 0.026	1.57 ± 0.029

The relative quantitation of DFR expression was calculated using the comparative threshold (C_T) method. The housekeeping gene elongation factor (EF-1α) was used as an endogenous reference, and ΔC_T was calculated by subtracting the average EF-1αC_T of gene of interest. This value was calculated for each sample (Tarocco, Navel and Ovale cDNA), then the comparative expression level of the DFR genes was given by the formula $2^{-\Delta\Delta C_T}$. For further details see Section 4.

mental growth conditions can influence the levels of anthocyanins of red oranges; in contrast, non-red *Citrus sinensis* (sweet orange) cultivars, such as Navel, Ovale and Valencia, do not produce these pigments. In this study we isolated the cDNA as well as the genomic clone of the DFR gene involved in the "late" steps of the anthocyanin biosynthetic pathway from both red and non-red orange cultivars. DFR cDNAs turned out to be identical in size and sequences, thus suggesting that the enzyme from blond and blood oranges should share the same structural and kinetic properties. A 2000 bp genomic DFR clone was also obtained from both pigmented and non-pigmented orange cultivars. Nucleotide sequence of blood and blond orange DFR genes showed very high identity values as only two mutations were found respectively within introns II and intron IV, as detailed in Fig. 3. It is important to note that the above mentioned mutations are not localized at the boundary of intron and exon. Therefore it is likely that the splicing process on the *dfr* primary transcript can occur equally in both cultivars. The promoter regions of *dfr* were also isolated from blood and blond orange cultivars (Fig. 4). Alignment of the red and non-red orange *dfr* 5′ upstream region (−986 bp upstream the initial codon ATG) showed more than 99% of identity as only one mutation was found, in a position where no consensus sequence for binding of transcription factors was detected. As significant differences were not discovered in the promoter regions of the blood and blond orange *dfrs*, we assume that they are equally capable of promoting *dfr* transcription.

Although red and non-red orange cultivars exhibit DFR genes characterized by identical encoding region as well as very similar non-encoding regions (introns and 5′ upstream region), their expression level is quite different, being about 250-fold less in the non-red orange cultivar than in the red one (Table 1). In accordance with this finding, we were able to measure the DFR activity only in the blood cultivar of Tarocco, whereas the Navel and Ovale blond cultivars did not show detectable DFR activity (Table 2). The SBF-1 and the TACpyAT elements might act as a transcriptional silencer for organ-specific expression (van der Meer et al., 1992; Lawton et al., 1991); however, these elements were detected in both promoters and cannot explain the difference of DFR expression in non-red oranges. The impaired expression of *dfr* in blond orange juice vesicles cannot even be ascribed to the presence of transposable elements within non-red cultivar DFR gene, as reported for Japanese morning glory (Inagaki et al., 1994) and carnation (Itoh et al., 2002) since the genomic *dfr* clone we isolated from blond orange was as long as that of the blood orange, and, as mentioned above, shared with red orange *dfr* very similar genomic sequence (except for two mutations within introns II and IV). Moreover, we also found that, both in the red and non-red orange genomes, the DFR gene is present as a single copy gene (Fig. 5). This result seems to exclude the possibility that red orange might express a higher amount of DFR because of an enhanced gene copy number. The UFGT expression was found to be crucial to specify the "colored" phenotype in grape skin (Boss et al., 1996). Therefore, Kobayashi et al. (2001) isolated and compared both the *ufgt* encoding region and the promoter sequences from white-skinned and their red-skinned sports, concluding that there were no differences between the two sequences, and supposing that a mutation of a regulatory gene controlling *ufgt* expression was responsible for the white phenotype. More recently, they definitively showed (Kobayashi et al., 2004) that a retrotransposon-induced mutation in *VvmybA1*, a *myb*-related gene regulating anthocyanin biosynthesis in the black-skinned grape cultivar, is associated with the loss of pigmentation in most of the white *Vitis vinifera* cultivars. Similarly, the DFR's low expression level observed in the blond oranges cultivars (Navel and Ovale), which might have a role in the phenotypic change from blood to blond orange, can be related to a mutation in a regulatory gene controlling the expression of the enzyme. We cannot exclude, however, that alternative mechanisms might also be involved, such as improper control of DNA methylation which might cause transcriptional repression (Curradi et al., 2002) either by directly interfering with the transcription factors binding to the blond orange *dfr* promoter or by attracting proteins that specifically bind methylated DNA, thus blocking access to factors required for *dfr* gene induction. In addition, here we report the successful expression of orange DFR cDNAs leading to an active DFR enzyme which converts dihydroquercetin to leucoanthocyanidin, confirming the involvement of the isolated genes in the anthocyanin biosynthesis. It is important to notice that, although DFR is a pivotal enzyme in the formation of plant pigments, very few successful in vitro expression systems have been available so far (Shimada et al., 2004; Martens et al., 2002; Fischer et al., 2003), thus making it impossible to obtain detailed biochemical analyses, and, in particular substrate specificity. Furthermore, as far as we know, this is the first report for in vitro expression of DFR of tree fruit flesh whose biochemical features might be very different from the ones of DFR of flowers or leaves as well as non-producing anthocyanin species. Consequently, our work lays the basis for deeper knowledge of DFR properties of fruit avoiding time-consuming protein purification procedures.

4. Experimental

4.1. General

Real time RT-PCR was performed in a Smart Cycler II PCR machine (Cepheid). Spectrophotometer quantification of cyanidin was carried out in a UV–VIS Shimadzu 1240 spectrophotometer. Trizol, Plant DNAzol, the DNA and RNA Quant-it assay kits, the Superscript RNAse H^- First-strand synthesis kit, Accuprime *Pfx* DNA polymerase, the SuperScript III Platinum two-step qRT-PCR kit, the pENTR-TOPO vector, the pEXP1-DEST Gateway vector, the Gateway LR Clonase Enzyme mix, the cell-free

protein system Expressway Plus were all obtained from Invitrogen. The Genome walker kit and the Advantage Genomic Polymerase mix were purchased from Clontech. Primers, Taqman probes and DNA sequences were obtained from MWG-Biotech. The Qiaquick gel extraction kit and the Qiaquick PCR purification kit were obtained from Qiagen. The Quickprep mRNA purification kit and Rediprime II DNA labeling system were purchased from Amersham. Nytran Supercharge nylon membranes were obtained from Schleicher & Schuell.

4.2. Plant material

Pigmented (Tarocco) and non-pigmented (Navel, Ovale) oranges [*Citrus sinensis* (L.) Osbeck] were harvested at commercial maturity from approximately 15-year old trees grown at a private farm in the territory of Francofonte (Italy). Freshly harvested orange fruits were peeled, chopped into 1 g samples, immediately frozen with liquid nitrogen and stored at −80 °C until used.

4.3. Extraction of genomic DNA and total RNA

Genomic DNA from both orange leaves and fruit juice vesicles was extracted using plant DNAzol as the extracting solution, whereas Trizol was employed to extract total RNA. DNA and RNAs were routinely quantified using the DNA and RNA Quant-it assay kits respectively.

4.4. cDNA synthesis and isolation of sweet orange DFR partial clone

Reverse transcription was achieved by using 2 μg of total RNA from Tarocco and Navel orange fruits and the Superscript RNAse H^- First-strand synthesis kit. To isolate DFR cDNA partial clone, two oligonucleotides designed by Joseph et al. (1998) on the basis of conserved amino acid sequences of several available DFRs were used: forward primer (5′(AG)AA(AG)TACATCCAICCIGTCAT3′); reverse primer (5′GA(AG)AA(CT)GA(AG)GTIAT(ACT)AA(AG)-CCIAC3′). The PCR reaction was carried out using the following "touch-down" conditions: 95 °C × 3 min, 55 °C × 30 s, 72 °C per 1 min (1 cycle); 95 °C × 15 s, 55 °C × 30 s, 72 °C per 1 min (1 cycle); 95 °C × 15 s, 53 °C × 30 s, 72 °C per 1 min (2 cycles); 95 °C × 15 s, 51 °C × 30 s, 72 °C per 1 min (2 cycles); 95 °C × 15 s, 49 °C × 30 s, 72 °C per 1 min (6 cycles); 95 °C × 15 s, 47 °C × 30 s, 72 °C per 1 min (6 cycles); 95 °C × 15 s, 45 °C × 30 s, 72 °C per 1 min (11 cycles); 72 °C × 5 min (1 cycle). The PCR experiment produced an amplification product of 204 bp, whose identity was confirmed by sequencing (Lo Piero et al., 2005).

4.5. Isolation of genomic and cDNA DFR clones

On the basis of the specific sweet orange DFR sequence obtained from the above described RT-PCR experiment, several steps of "genome walker" were performed to isolate both the 5′ and the 3′ DNA ends of the DFR gene from both blond (Navel) and blood (Tarocco) orange juice vesicles. Then, forward and reverse primers bordering the DFR gene were designed, (DFR5′UTR-5′CAAGAGTTTCGAAAT-TCCTTGATC3′), (DFRPOL2-5′AACACAATAATTCT-ACCTGCGTTA3′) and used in the following PCR strategies: (A) cDNA as template (200 ng): 94° × 2 min (1 cycle), 94 °C × 40 s, 55 °C × 40 s, 68 × 2 min (25 cycles), 72°C × 20 min (1 cycle); (B) genomic DNA (200 ng) as template: 94° × 5 min (1 cycle), 94 °C × 40 s, 55 °C × 40 s, 68 × 2 min (35 cycles), 72 °C × 20 min (1 cycle). Accuprime *Pfx* DNA polymerase was used to isolate the orange DFR cDNA as well as the DFR genomic clones from pigmented (Tarocco) and non-pigmented (Navel) oranges. The PCR products were purified, cloned into the PCR4-TOPO vector and sequenced. The experiments were repeated at least three times on independently isolated nucleic acid preparation in order to discriminate authentic sequence mutations from procedure-linked mistakes.

4.6. Southern-blot analysis

Genomic DNAs (10 μg) isolated from Tarocco and Navel orange leaves were digested overnight with 5 U/μg DNA of the following restriction enzymes: *Ava*II, *Eco*RV, *Eco*RI and *Bst*NI. Fragments were concentrated by sodium acetate-ethanol precipitation, accurately quantified using the DNA Quant-it kit and separated by electrophoresis on 0.7% agarose gel. After denaturation and neutralization according to standard procedures (Sambrook and Russel, 2001), DNA fragments were transferred to Nytran Supercharge nylon membrane and probed overnight with radioactively labeled full-length DFR cDNA in hybridization buffer containing: 6X SSC, 5X Denhardt's reagent, 0.5% sodium lauryl sulfate, 100 μg/ml salmon sperm DNA, 50% formamide. The membrane was washed at high stringency (0.1% SSC, 0.1% SDS, at 65 °C for 2 h) and exposed to X-ray film. The 1017 bp DFR cDNA clone was radioactively labeled using ^{32}P(dCTP) and the Rediprime II DNA labeling system.

4.7. Isolation of the 5′ upstream region

Blood and blond orange DFR 5′ upstream regions were isolated by a double step of "genome walker". Firstly, pools of uncloned, adaptor-ligated genomic DNA fragments were constructed starting from very clean and high average molecular weight sweet orange DNA (Tarocco and Navel). Then, for the first "genome walker" step, consisting of two consecutive PCR reactions (primary and secondary PCR), the amplification was performed with a pair of reverse primers designed within the DFR coding region (DFR-GSP1- rev 5′CTAAATCGGCTTTCCATAAAGTCAGGT3′, primary PCR; DFR-GSP2-rev 5′TTTTGTTATCAGG-ATCGCGAACAGTAG3′, secondary PCR) and two outer forward adaptor primer (AP1 5′GTAATACGACTCA-CTATAGGGC3′, primary PCR; AP2 5′ACT-

ATAGGGCACGCGTGGT3′, secondary PCR). The major PCR product, 593 bp long, was gel-purified, cloned into the PCR4-TOPO vector and sequenced. The fragment was identified as a 5′ flanking region of the DFR coding region since it overlapped the known DFR sequence for 100 bp. To go further upstream, the second step of "genome walking" was carried out with a pair of GSP reverse primers designed within the DFR sequence obtained from the first step (DFR-GSP1/2-rev 5′ATCTGCCCACATGCCGAAAATATTTGA3′, primary PCR; DFR-GSP2/2-rev 5′GAAAATCTTGCAGCACACGTAAATTCC3′, secondary PCR) and two outer forward adaptor primer (AP1, primary PCR; AP2, secondary PCR). A 650 bp fragment was obtained, gel-purified, cloned and sequenced. The 800 bp band was identified as the 5′ upstream region since it overlapped the known sequence for 157 bp. The Advantage Genomic Polymerase mix, which is specifically developed for LD-PCR (long distance-PCR) using genomic template, was used for both primary and secondary PCR following the experimental conditions suggested by the manufacturer. The location and sequence of gene specific primers are indicated in Fig. 3.

4.8. Measurement of dfr expression by real-time quantitative RT-PCR

Real-time PCR, Taqman assays, were performed using the SuperScript III Platinum two-step qRT-PCR kit. Pure mRNA was prepared from freshly harvested juice vesicles using the Quickprep mRNA purification kit. Reverse transcription of mRNA (500 ng) was achieved by following the manufacturer's protocol. Primers and probe were: for-Citrus-DFR (5′GCTGTTCGTGCTACTGTTC3′), rev-Citrus-DFR (5′GTTTCCCTCTTCGGCTAAATC3′), DFR-probe (5′FAM-AAGGCAAGCACTCACCTGACTTTATGGAAA-3′TAMRA). The relative quantitation of DFR expression between blood and blond oranges was calculated using the comparative threshold (C_T) method (Heid et al., 1996). Three independent triplicates of quantitative PCR experiments were performed for each gene to generate an average C_T and to calculate standard deviation. The housekeeping gene elongation factor (EF-1α), which has been reported to be constitutively expressed (Mahe et al., 1992), was used as an endogenous reference, and ΔC_T was calculated by subtracting the average EF-1α C_T from the average C_T of gene of interest. This value was calculated for each sample (Tarocco, Navel and Ovale cDNA), and then the comparative expression level of the DFR genes was given by the formula $2^{-\Delta\Delta C_T}$ where $-\Delta\Delta C_T$ was calculated by subtracting the ΔC_T of the baseline from the ΔC_T of the sample, where the baseline represents the minimum level of expression (Navel). The dynamic range of both DFR and EF-1α was determined by monitoring the variation of ΔC_T with template dilution; efficiency was very similar for both primer/probe systems. Elongation factor forward and reverse primers were: for-Citrus-EF (5′CACCACCCCCAAGTACTC3′), rev-Citrus-EF (5′GTTGTCACCCTCGAAACC3′), EF-probe (5′FAM-AAGGTTGGATACAACCCCGAGAAAGTCCCA3′-TAMRA). For each triplicate, 5 ng of cDNA was added to a final volume of 25 μl with a final concentration of 1X Platinum two-step qRT-PCR master mix, 100 nM each primer and 100 nM probe. The cycling program was: 50 °C × 2 min (1 cycle), 94 °C × 2 min (1 cycle), 94 °C × 15 s, 55 °C × 30 s, 72 °C × 30 s (45 cycles). Negative controls without reverse transcriptase were routinely included. The experiments were repeated at least three times on independently isolated mRNA preparation.

4.9. Enzyme extraction

Freshly harvested orange fruits (Tarocco and Navel) were peeled, chopped into small pieces and homogenized using a waring blender with extraction buffer (0.1 M borate-HCl pH 8.8 containing 0.4 M NaCl, 10% glycerol, 20 mM sodium ascorbate, 2.5% polyvinylpyrrolidone) in a 1:2 w/v ratio. The homogenate was filtered through two layers of cheesecloth and centrifuged at 10,000g × 10 min to remove solid particles. The pellet was discarded whereas the supernatant was further centrifuged at 150,000g × 30 min. Proteins of the soluble fraction were precipitated by 70% saturated ammonium sulfate, collected by centrifugation (12,000g × 30 min) and resuspended in 1.5 ml of extraction buffer. The sample was then desalted by dialysis against a total of two 4 l of extraction buffer changes for 10 h. All procedures were carried out at 4 °C. Protein concentration was determined according to the method of Bradford (1976) using bovine serum albumin as standard. DFR activity on crude extract of orange fruit was assayed as described below. The experiments were repeated four times on independently prepared crude extracts.

4.10. In vitro expression of DFR

The DFR gene was PCR-amplified from TOPO-plasmid *dfr* clone with the Accuprime *Pfx* DNA polymerase PCR kit. The following primers (forward, 5′CACCATGGGCTCTATAGCTGAGACTG3′, introducing the 5′ underlined CACC tail suitable for directionally cloning in pENTR-TOPO; reverse, 5′TTATATGGATACTTCGCTGAC3′) were used in a PCR experiment performed according to the manufacturer's instructions. The PCR product was purified and cloned into the TOPO-pENTR vector; once the ligation reaction was performed the TOPO-pENTR-DFR construct was used to transform into *E. coli* One Shot TOP10 chemically competent cells. Positive clones were identified both by PCR analysis of the insert and by sequencing with M13 forward and reverse primers. The DFR coding sequence was then transferred from the entry construct (pENTR-DFR) into the pEXP1-DEST Gateway vector using the Gateway LR Clonase Enzyme mix based on the site-specific recombination properties of bacteriophage lambda. After the recombination reaction, the DFR-pEXP1-DEST was used to transform into *E. coli* library

efficiency DB3.1 competent cells. The presence and identity of the insert was tested both by PCR analysis and by sequencing with T7 promoter and T7 reverse primers. In vitro expression of functionally active DFR was achieved by using the cell-free protein system Expressway Plus by incubating the plasmid (DFR-pEXP1-DEST) in the IVPS Plus *E. coli* extract for 4 h at 25 °C to promote proper protein folding. A negative control was also performed by incubating the *E. coli* extract with empty plasmid (pEXP1-DEST).

4.11. DFR enzyme assay

The DFR assay was performed according to a slight modification of the method described in Shimada et al. (2004). The reaction mixture (final volume 500 μl) containing 0.05 M Tris–HCl pH 7.5, 7.5 μM (±) dihydroquercetin, 2 mM NADPH, 1 U glucose-6-phosphate dehydrogenase, 6 mM glucose-6-phosphate and enzyme extract (10–30 μg) was incubated at 25 °C for 4 h. The leucoanthocyanidin was extracted twice with ethyl acetate, which was subsequently evaporated to dryness by overnight incubation at 50 °C. The residue was dissolved in 20 μl of butanol:HCl (95:5 v/v) and boiled for 5 min. Cyanidin was spectrophotometrically quantified ($\varepsilon_{550} = 29600$) against a sample blank in which the enzyme extract was added at the end of the incubation, immediately before ethyl acetate extraction.

4.12. PCR product analysis, cloning and sequencing

PCR products were separated by 1.1% agarose gel containing 0.5 μg/ml ethidium bromide. The Qiaquick gel extraction kit and the Qiaquick PCR purification kit were used to extract PCR fragments from the agarose gels and PCR amplification reaction mixture, respectively. The amplified fragments were cloned into PCR4 TOPO vector and sequenced by the fluorescence detection method, using both T7 and T3 as primers. A comparison of the deduced amino acid sequences was performed by using the multiple sequence alignment program CLUSTALW 1.8. Transcription factor binding sites were searched for using the MatInspector program (Genomatix available from the web site at http://www.genomatix.de) with threshold of 80.0 points for matrix similarity and 100.0 points for core similarity. The nucleotide sequences reported in this paper were submitted to Genbank under accession numbers: **AY519363** (DFR, complete cDNA), **DQ084722** (Navel DFR genomic clone), **DQ084723** (Tarocco DFR genomic clone), **CF972317** (DFR partial clone), **AY498567** (EF-1α partial coding sequence).

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.phytochem.2006.01.025.

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