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# Flavonoid metabolism and gene expression in developing olive (Olea europaea L.) fruit

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# Flavonoid metabolism and gene expression in developing olive (*Olea europaea L.*) fruit

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**Abstract**
The expression pattern of six genes (*phenylalanine-ammonia-lyase*, PAL, *chalcone synthase*, CHS, *flavanone 3-hydroxylase*, F3H, *dihydroflavonol 4-reductase*, DFR, *anthocyanidin synthase*, ANS, UDP-*glucose-flavonoid 3-O-glucosyltransferase*, UFGT) was studied, together with the accumulation of total flavonoids and anthocyanins in developing olive (*Olea europaea* L.) fruit. Flavonoid concentration showed the highest values in young fruit whereas anthocyanins accumulated at ripening, in particular in epicarp tissue, concurrently with an up-regulation of UFGT. PAL, CHS, F3H, and UFGT were expressed at the early stages of fruit development when DFR and ANS transcripts were not detected. DFR was induced in the epicarp at the onset of ripening and color change, while ANS transcripts were extremely abundant at a more advanced stage. A coordinated up-regulation of the genes involved in the last steps of anthocyanin biosynthesis was observed in ripe olives. These results suggest that DFR and ANS, together with UFGT, might represent key elements in the regulation of anthocyanin biosynthesis in olives, and that the expression pattern of these two genes could be used to monitor, at the molecular level, the evolution of ripening in fruits of this species.

**Keywords:** *Anthocyanins, gene expression, flavonoid, olive ripening, transcript accumulation*

## Introduction

Phenolic compounds represent a complex mixture in both olive fruits and oil, their derived product. These compounds are responsible for the anti-atherogenic and anti-cancerogenic effects, and for the antioxidant properties of olive oil, one of the major constituents of the Mediterranean diet (Tripoli et al. 2005; Hashim et al. 2008; Llorente-Cortes et al. 2010).

Several parameters affect olive fruit phenolic content and these include genotype, pedoclimatic conditions, agronomic techniques (e.g. irrigation, fertilization), and the developmental stage of the drupe (Briante et al. 2002; Vinha et al. 2005; Aganchich et al. 2007; Tognetti et al. 2008; D'Andria et al. 2009; Sgromo et al. 2010; Assab et al. 2011; Rewald et al. 2011). Flavonoids, including flavonols, anthocyanins, and tannins, are an important class of phenolic compounds since they have many biological functions (Schijlen et al. 2004). Anthocyanins but also flavonols, which act as their co-pigments, are responsible for specific colors of plant tissues, and the accumulation of these pigments in fruit (together with the degradation of chlorophyll) is an important determinant of maturation and ripeness in several species including olive.

The biosynthetic pathway of anthocyanins has been extensively investigated in different species, such as petunia, snapdragon, maize, and grape (Quattrocchio et al. 1993; Deboo et al. 1995; Boss et al. 1996; Bradley et al. 1998). Two classes of genes are required for anthocyanin production, the structural genes that encode the enzymes that directly participate in anthocyanin synthesis and, more in general, in flavonoid formation, and the regulatory genes that control the transcription of structural genes. Transcriptional control plays an important role in regulating overall flavonoid biosynthesis (Koes et al. 2005), and the activities of enzymes in the anthocyanin pathway are under developmental and tissue-specific control (van Tunen et al. 1988).

In olive, preliminary studies on basic mechanisms regulating phenolic compounds have been recently performed although the molecular mechanisms

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involved in the regulation of flavonoid biosynthesis is still unknown. Using large-scale transcriptome profiling, specific genes involved in flavonoid and terpenoid pathways have been shown to be differentially expressed during fruit development (Alagna et al. 2009; Galla et al. 2009). The temporal expression of two genes (chalcone syntase, CHS, and anthocyanidin synthase, ANS) was analyzed in olive fruit at five different stages without studying tissue-specific expression (Ferrante et al. 2004). Besides these works, no studies have been performed to identify genes playing a major role in the regulation of the entire flavonoid biosynthetic pathway in olive fruit. This knowledge would have important agronomic implications. Indeed, understanding the molecular regulation of flavonoid, in general, and in particular, anthocyanin, biosynthesis could be useful to manage olive harvest, enhance quality, and maximize yield. In addition, the isolation of flavonoid gene sequences and the analysis of their expression are essential to identify the agronomic and environmental factors regulating their expression throughout olive fruit development. This information can be used to set up strategies that eventually modulate flavonoid content in olives and, consequently, improve, health-related properties of olive oil.

Based on these considerations, the present work was aimed at elucidating some aspects of flavonoid metabolism, and identifying the gene(s) that may play a key role at the transcriptional level in the biosynthetic pathway of anthocyanins in olive fruits.

## Materials and methods

### *Plant material*

Fruit was sampled in 2008 from *Olea europaea* (*L.*) (cv *Leccino)* trees cultivated near Lucca (Italy) at 4 weeks after flowering (WAF) (before pit hardening), 9 WAF (after pit hardening), 12 WAF (mature fruit, green epicarp pigmentation), and 16 WAF (ripe fruit, about 50% epicarp pigmentation change). At 4 and 9 WAF, the entire pericarp was collected, whereas epicarp and mesocarp were collected separately at 12 and 16 WAF. All samples were frozen with liquid nitrogen and stored at $-80^{\circ}$C until use. All analyses were performed using three replicates consisting of a pool of 10 homogeneous fruits.

### *RNA extraction*

Fruit tissues were ground in liquid nitrogen using a mortar and pestel. RNA was isolated using the RNeasy Plant RNA Isolation Kit (Qiagen, Venlo, Netherlands). RNA was eluted with 50 $\mu$l of dd$H_2O$ and stored at $-80^{\circ}$C until use. The functional quality of the extracted RNA was evaluated by agarose gel electrophoresis with ethidium bromide staining. Purity of RNA was checked measuring absorbance ratio (280/260 nm) with values ranging from 1.6 to 1.9.

### *Isolation of UFGT and F3H sequences*

Reverse Transcriptase (RT)-PCR was used to isolate *UDP-glucose-flavonoid 3-O-glucosyltransferase* (UFGT) cDNA from total RNA of fruit at 16 WAF. RNA was treated with DNase using the Turbo DNA-free kit. First-strand cDNA was synthesized using M-MLV Retrotranscriptase following the manufacturer's instructions (Life Technologies, Carlsbad, USA). The amplification mix was prepared using the GoTaq protocol (Promega, Fitchburg, USA). For PCR reaction, sense (5′-TGGGTGTCATTTTGGACYGC-3′) and antisense (5′-GGCCTGCAAATCATRGG-3′) primers were used based on the alignments of some UFGT cDNA sequences from different species present in the NCBI database. The following cycle conditions were used: 30 s at 94°C, 35 s at 50°C, 30 s at 72°C for 35 cycles. An amplified fragment of approximately 710 bp was cloned in Teasy vector (Promega), sequenced, and compared at the nucleotide sequence level using Blast analysis available on the NCBI website.

*Flavanone 3-hydroxylase* (F3H) EST was isolated from forward- and reverse-subtracted libraries obtained by Galla et al. (2009). The complete analysis was carried out using CLC Combined Workbench 3 software. The EST sequences of *phenylalanine-ammonia-lyase* (PAL), *chalcone synthase* (CHS), *dihydroflavonol 4-reductase* (DFR), and *anthocyanidin synthase* (ANS) were downloaded from the NCBI website. Multiple alignments were performed using ClustalW (http://align.genome.jp/).

### *Gene expression analysis*

Semi-quantitative RT-PCR analysis was carried out according to the manufacturer's instructions (Reverse Transcription System, Promega). A pair of primers was designed for each of the six flavonoid biosynthetic genes analyzed (Table I).

Quantum 18S RNA (Universal kit, Life Technologies Inc.) was used as housekeeping gene. The following cycle conditions were adapted for each gene following the instructions of the kit. The PCR products were separated by electrophoresis on 1.5% agarose gels. Amplification mix was prepared using GoTaq protocol (Promega). The amplification products were scanned and quantified using the Quantity One software (BioRad, Hercules, USA). The relative expression level for each target gene was determined as band intensity relative to the housekeeping gene (transcript ratio).

Table I. Genes and primers used in semi-quantitative PCR analyses.

| Gene | Primers |
|---|---|
| PAL | F 5′-CGCCGTGCTTACCCCTCCGTGG-3′ |
| | R 5′-TGAAGCCAAGCCAGAACCAACAGCC-3′ |
| CHS | F 5′-TCATGATGTACCAACAGGGCTGCTTCG-3′ |
| | R 5′-GGCCGCTCCACCCCAATCACC-3′ |
| DFR | F 5′-GCTTCTGGCTTCATCGGCTCATGG-3′ |
| | R 5′-CTCCTTCACATCCGTGGATTGCTTCGT -3′ |
| F3H | F 5′-TCCCATTGCTTAGATAATGACCATGGTCC-3′ |
| | R 5′-GTGAATTTCTATCCAAAATGCCCGCAG |
| ANS | F 5′-AGGTCTAACAAGCAAAATACTATCGGCAC-3′ |
| | R 5′-CCTCACCTTCTCTCTTTATTCACAAGCCCTC-3′ |
| UFGT | F 5′-TCGTCAGTGGAGATTCAGACCAGGAATGC-3′ |
| | R 5′-TCACCAGTTTCGAGTACTTCTGCCAATGC-3′ |



### *Total anthocyanin quantification*

Frozen fruit tissue (100 mg) was ground in a pre-chilled mortar and extracted into 5 ml methanolic HCl (1%) and incubated overnight at 4°C in darkness. After centrifugation at 5000 RCF for 5 min, the supernatant was filtered and used for spectroscopic analysis. Concentration of anthocyanins was expressed as cyanidin-3-glucoside equivalents determined with a spectrophotometer at 535 nm using an extinction coefficient of 29.600. The entire procedure was repeated three times for each developmental stage analyzed.

### *Total flavonoid quantification*

One gram of ground olive tissues was mixed with 40 ml of hexane and agitated for 4 min; the upper phase was recovered and the extraction was repeated twice successively with the lower phase. Flavonoid compounds were extracted with 80 ml of 80% (v/v) methanol containing 0.04% (w/v) sodium metabisulphite. The mixture was agitated for 5 min, and then centrifuged for 5 min at 3000 RCF to separate the hydromethanolic phase. This procedure was repeated three times using the same tissue; the hydromethanolic phases were then combined and filtered. Flavonoid content was measured using different dilutions of catechins as a standard. The test was performed in a 5-ml final solution containing: 500 $\mu$l MilliQ water, 150 $\mu$l $NaNO_2$ 5%. After 5 min, $AlCl_3$ 10% was added. The reaction was ended with 1 ml of 1M NaOH after 6 min of incubation, and absorbance measured at 510 nm. The analysis was repeated three times for each developmental stage.

### *Statistical analysis*

Mean values and correspondent standard deviations of three RT-PCRs were calculated using the Quantity One software (BioRad). Statistical analysis was performed using one way-ANOVA and LSD as *post-hoc* test ($P < 0.05$). The same descriptive parameters were determined for total flavonoid content using three independent fruit pools for each developmental stage analyzed.

## Results

### *EST isolation and sequence analyses*

To isolate a portion of the UFGT cDNA sequence, degenerate primers through alignment between cDNA sequences from *Vitis vinifera* and *Forsythia x intermedia* were designed. The 5′ primer covered position 408–428 in the *Forsythia x intermedia* complete coding sequence, while the 3′ primer covered position 986–1006. PCR analyses performed using these primers yielded a single band of 711 bp. Amplification products were cloned in Promega Teasy vector and four clones containing a 711–bp fragment were sequenced and found to be identical. Using ClustalW software, the cDNA fragment exhibited an aligned score of 30.3 when aligned to the correspondent gene of *Forsythia x intermedia*. The deduced protein exhibited for the same parameter a value of 75.9 in comparison with the ortholog protein of *Forsythia x intermedia* (Figure 1).

UFGT partial sequence was deposited in the NCBI database with accession number EU562294. Partial cDNA sequences of the other flavonoid genes analyzed were compared with homolog sequences present in the NCBI databank. The olive sequence of DFR showed 90% identity to the homolog sequence of *Forsythia x intermedia*. For F3H, Blast analysis showed close similarities between this partial sequence and the ones from other species such as *Camelia sinensis* (81% identity), *Sinningia cardinalis* (80% identity), *Gossypium hirsutum* (80% identity), *Malus domestica*, *Prunus persica*, *V. vinifera* (78% identity with each). Olive PAL cDNA showed highest similarity with hortologs in several other species, such as *Osmathus flagrans* (95% identity), *Nicotiana tabacum* (81% identity), *Ipomea batatas* (79% identity). Interestingly, the olive CHS showed the highest (78% identity) similarity with the *V. vinifera* hortolog, while ANS displayed the highest similarity (89% identity) with the correspondent sequence of *Forsythia x intermedia*.

### *Expression pattern of flavonoid genes*

Expression of the six selected genes was determined in fruit sampled at four developmental stages (Figure 2).

For the last two stages, epicarp and mesocarp were analyzed separately. Data indicated that the considered genes are differentially expressed during olive fruit development. PAL expression was high at the

```
_Olea_europaea_L.          --------------------------------------------------
_Forsythia_x_intermedia    MAIHSHIGVLAFPFGTHAAPLLTLVRRLVLDSSSQGITFSFFNTAKSNCA

_Olea_europaea_L.          --------------------------------------------------
_Forsythia_x_intermedia    IFSGQEFDNIKAYDVWDGTHEGEAFTGSNILEAMQLFLAATPGNFEKVMK

_Olea_europaea_L.          --------------------------------WVSFWTASPSSLSAHMCT
_Forsythia_x_intermedia    EAEVKNGMKISCLLSDAFLWFTCDLAEERGIPWVSFWTAASCSLSAHMYT
                                                          *******:..****** *

_Olea_europaea_L.          DQIWSMMRSIGAAEREEKTLSFLPGMSSVRFSDLPGEILPENSESPLAIM
_Forsythia_x_intermedia    DQIWSLMRSTGTAKTEEKTLSFVPGMTSVRFSDLPEEILSDNLESPLTLM
                           *****:*** *:*: *******:***:******** ***.:* ****::*

_Olea_europaea_L.          IYKMVQKLPKSTAVVINSFEEIYTLIKNDLKSKFQNFLDIQLSILSEDPS
_Forsythia_x_intermedia    IYKMVQKLSKSTAIVVNSFEEIDPVITNDLKSKFQNFLNIGPSILSS-PT
                           ********.****:*:****** .:*.***********:*  ****. *:

_Olea_europaea_L.          IVSGDSDQECLSWLEKQRHASVVYISFGTVAEAQPEELAALAEVLETGEF
_Forsythia_x_intermedia    LSNGDSGQECLLWLEKQRHASVIYISFGTVITPQPREMAGLAEALETGEF
                           : .***.**** **********:*******  .**.*:*.***.******

_Olea_europaea_L.          PFLWSMRDNAKKLLPEGFLNRTSKFGMIVSWAPQLKVLENPSVGVHMTHG
_Forsythia_x_intermedia    PFLWSLRDNAMKLLPDGFLDRTSKFGMIVSWAPQLKVLENPSVGAFITHC
                           *****:**** ****:***:************************..:**

_Olea_europaea_L.          GWNSVLESISCEVPMICRP-----------------------------
_Forsythia_x_intermedia    GWNSILESISFGVPMICRPFFGDQNLNSKMVEDVWKIGVRLEGGVFTKNG
                           ****:*****  *******

_Olea_europaea_L.          --------------------------------------------------
_Forsythia_x_intermedia    TIEALHSVMLNETGKAIRENINKLKRKAQNAVKFDGTSTKNFRALLELIK
```

Figure 1. ClustalW output for the alignment of the UFGT protein sequences from *Olea europaea* (partial) and *Forsythia x intermedia* (complete). Symbol ''*'' means identical aminoacids, '':'' means conserved substitutions, ''.'' means semi-conserved substitutions.

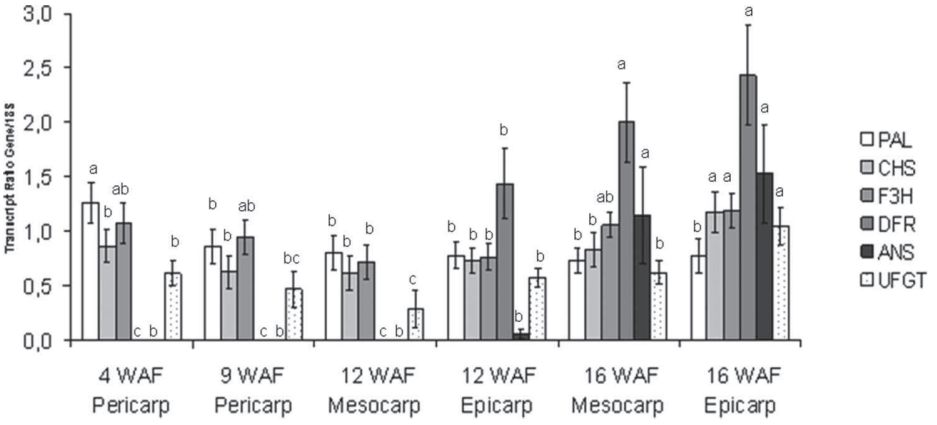


Figure 2. Expression of PAL, CHS, F3H, DFR, ANS, and UFGT genes in olive (*Olea europaea)* fruit at four different development stages (the weeks after flowering, WAF, are indicated). The values indicate the ratio between the specific gene and the 18S housekeeping gene. Within each gene, different letters indicate statistically significant differences between developmental stages according to *t*-test LSD. Standard deviations are indicated for each mean value.

first sampling date (4 WAF) and then slightly decreased, remaining stable throughout development. No difference between epicarp and mesocarp PAL transcript accumulation was observed in ripening fruit (12 and 16 WAF). CHS, the first gene of the flavonoid biosynthetic pathway, was expressed at 4 WAF and did not show any significant transcriptional changes during fruit development, with the exception of the last sampling date (16 WAF) when a significant increase was detected in the epicarp. A similar trend was observed for F3H and UFGT that was transcribed in young fruit, and showed an enhanced expression in both mesocarp and epicarp at 16 WAF. A different expression pattern was

detected for DFR and ANS. In fact, neither was expressed at 4 and 9 WAF in the pericarp, and at 12 WAF in the mesocarp. Specific mRNA accumulation (more abundant for DFR) was observed in the epicarp starting at 12 WAF, and marked increases were evident for transcripts of both genes in both tissues sampled at 16 WAF (Figure 2).

### *Flavonoid and anthocyanin quantification*

Flavonoids were present in fruit tissues at the early stages of growth and development (Figure 3A).

At 9 WAF, their level dramatically increased reaching the highest concentration; a significant decrease was observed thereafter. Levels were similar in epicarp and mesocarp at both of the last developmental stages analyzed, with an increasing trend (although statistically not significant) in the epicarp at 16 WAF. Anthocyanin concentration was low in the pericarp at 4 and 9 WAF, and in both mesocarp and epicarp at 12 WAF (Figure 3B). At ripening, the epicarp, but not the mesocarp, showed a significant increment in anthocyanin concentration.

## Discussion

This work was aimed at determining what genes play a major role in the transcriptional regulation of the flavonoid pathways in olive fruit. PAL is a key enzyme for the synthesis of phenylpropanoids since it catalyses the deamination of phenylalanine, thus producing *trans*-cinnamate. This is a common passage to the biosynthetic pathways of all polyphenols that show the highest accumulation in the early developmental stages of many fruits including olive (Alagna et al. 2009). In *Vitis* and *Arabidopsis*, PAL is encoded by multigene families (Sparvoli et al. 1994; Olsen et al. 2008), and gene family members may be differentially regulated in plant tissues. As far as we know, no data were previously reported on PAL expression in olive fruit where specific transcript accumulation was high at four WAF and then decreased during further fruit development. A higher

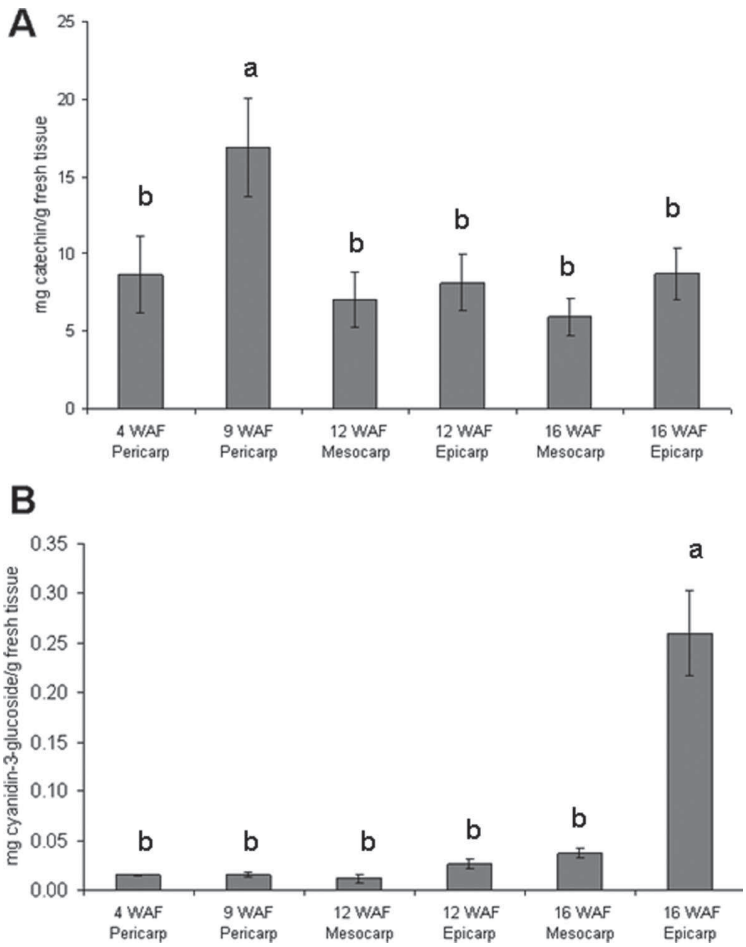


Figure 3. Total flavonoid (A) and anthocyanin (B) concentration in olive (*Olea europaea*) cv *Leccino* fruit development determined at four different developmental stages (the weeks after flowering, WAF, are indicated). Different letters in each panel indicate statistically significant differences between developmental stages according to *t*-test LSD Standard deviations are indicated for each mean value.

accumulation of PAL transcripts during the early developmental stages could be related to the high concentration of phenol compounds (e.g., hydroxytyrosol and other low-molecular weight phenols) detected in young fruits (Agati et al. 2005). The flavonoid content of olive fruit appears to be quite low if compared with other phenolic classes (e.g. secoiridoids such as oleoeuropein) (Romani et al. 1999). The accumulation pattern showed a high flavonoid concentration in young fruit when CHS, the first committed enzyme of the flavonoid pathway, is actively transcribed: this behavior is similar to that observed in citrus (Moriguchi et al. 2001) and apple (Renard et al. 2007) fruit. In general, previous studies performed in other crop species showed that flavonoids and their glycosides accumulate until tissues and organs cease to grow and then start decreasing (Kamsteeg et al. 1980; Castillo et al. 1992). Our data on CHS and F3H expression at ripening confirm the findings of Galla et al. (2009) who reported a coordinated up-regulation of key genes (including CHS and F3H) related to anthocyanin biosynthetic pathway at the veraison (onset of ripening) stage. Interestingly, UFGT followed a similar pattern of expression, since it appeared activated at the earliest developmental stage analyzed (four WAF), and remained stable during the subsequent stages of fruit development. At ripening, it showed an up-regulation in both mesocarp and epicarp. This confirms previous data showing that flavonoid genes are differentially regulated during fruit development, and that some differences among species are present. Studies in grape berry showed that the expression of several genes, with the exception of UFGT, was detected at 4 WAF followed by a reduction from 6 to 8 WAF (Boss et al. 1996). UFGT appeared to be regulated independently and activated only at veraison, suggesting that, in grapes, the major control point in this pathway is at the final step of anthocyanin biosynthesis (Boss et al. 1996). Besides the increase of expression in both mesocarp and epicarp at ripening, we observed that UFGT is also transcribed in young fruit when anthocyanins are not accumulating, suggesting that this gene might be involved in other processes in addition to anthocyanin synthesis. In strawberry, the presence of several genes encoding UDP-requiring enzymes involved in the biosynthesis of different products has been hypothesized (Manning 1998). Besides the ripening-associated anthocyanin formation, glycosyltransferases may catalyze the addition of sugar moieties to other phenylpropanoids, such as flavonols and flavanols. Whether or not this is the case for young olive fruit remains to be elucidated. Other hypothesized roles of UDP-dependent enzymes are related to the regulation of auxin concentration through glycosylation. Ester-conjugated IAA has been detected in strawberry receptacles, and glycosylation could be a mechanism for regulating the concentration of free IAA in the fruit (Manning 1998). Thus, we cannot exclude that in olive fruit an early expression of UFGT might be related to similar processes, while the increase of its expression (together with that of other flavonoid genes) regulates anthocyanin biosynthesis at ripening.

In both strawberry and grape berries, a general up-regulation of flavonoid genes occurs in immature fruit and the same set of genes, with the addition of UFGT, is induced at veraison (Boss et al. 1996; Manning 1998). Our results showed that DFR and ANS were not expressed at the early stages of fruit development, and started to be transcribed only at the onset of ripening. Up-regulation of these genes was also detected by Galla et al. (2009) and Alagna et al. (2009) in their genomics approaches to study olive fruit development. According to transcript profiling, ANS and DFR appear to be key elements of regulation for the activation of anthocyanin biosynthesis in olive fruit. Tsuda et al. (2004) suggested that DFR (together with CHS) may represent the key regulatory genes in the process of anthocyanin biosynthesis in mature red-skinned peach fruit. At 12 WAF, ANS and DFR were expressed only in the epicarp where the color change starts well in advance compared with the mesocarp when olives approach the ripening stage (Figure 3B). Taken together, these data reinforce the hypothesis of a crucial role played by DFR and ANS genes that, together with UFGT, could be used to monitor, in terms of specific transcript accumulation, the evolution of ripening in olive fruit. It is known that in grape and apple, polyphenol biosynthetic genes are regulated by different transcription factors (Takos et al. 2006; Bogs et al. 2007). A large amount of evidence has accumulated regarding transcription factors and the corresponding cis-acting elements that control the expression of phenylpropanoid biosynthetic genes (Deluc et al. 2008; Hichri et al. 2011). In olive fruit, no orthologs of these transcription factors have been isolated yet. However, it is possible that a similar combinatorial interaction of these types of transcription factors regulates anthocyanin biosynthesis. Based on the results presented here, we hypothesize the existence, also in olive, of several types of transcription factors differentially involved during fruit development and ripening in the modulation of flavonoid gene expression.

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## References


Aganchich B, Tahi H, Wahbi S, Elmodaffar C, Serraj R. 2007. Growth, water relations and antioxidant defence mechanisms of olive (Olea europaea L.) subjected to Partial Root Drying (PRD) and Regulated Deficit Irrigation (RDI). Plant Biosyst 141: 252–264.

Agati G, Pinelli P, Cortes Ebner S, Romani A, Cartelat A, Cerovic ZG. 2005. Nondestructive evaluation of anthocyanins in olive (*Olea europaea*) fruits by in situ chlorophyll fluorescence spectroscopy. J Agric Food Chem 53: 1354–1363.

Alagna F, D'Agostino N, Torchia L, Servili M, Rao R, Pietrella M, et al. 2009. Comparative 454 pyrosequencing of transcripts from olive genotypes during fruit development. BMC Genomics 10: 399.

Assab E, Rampino P, Mita G, Perrotta C. 2011. Heat shock response in olive (*Olea europea L.*) twigs: Identification and analysis of a cDNA coding a class I small heat shock protein. Plant Biosyst 145: 419–425.

Bogs J, Jaffe VFW, Takos AM, Walker AR, Robinson SO. 2007. The grapevine transcription factor VvMYBPA1 regulates proanthocyanidin synthesis during fruit development. Plant Physiol 143: 1347–1361.

Boss PK, Davies C, Robinson SP. 1996. Analysis of the expression of anthocyanin pathway genes in developing *Vitis vinifera L* cv *Shiraz* grape berries and the implications for pathway regulation. Plant Physiol 111: 1059–1066.

Bradley JM, Davies KM, Deroles SC, Bloor SJ, Lewis DH, 1998. The maize Lc regulatory gene up-regulates the flavonoid biosynthetic pathway of Petunia. Plant J 13: 381–392.

Briante R, Patumi M, Limongelli S, Febbraio F, Vaccaro C, Di sale A, et al. 2002. Changes in phenolic and enzymatic activities content during fruit ripening in two Italian cultivars of *Olea europaea L.* Plant Sci 162: 791–798.

Castillo J, Benavente O, Delrio JA. 1992. Naringin and neohesperidin levels during development of leaves, flower buds, and fruits of citrus-aurantium. Plant Physiol 99: 67–73.

D'Andria R, Lavini A, Morelli G, Sebastiani L, Tognetti R. 2009. Physiological and productive responses of *Olea europaea L.* cultivars Frantoio and Leccino to a regulated deficit irrigation regime. Plant Biosyst 143: 222–231.

Deboo GB, Albertesen MC, Taylor LP. 1995. Flavanone 3-hydroxylase transcripts and flavonol accumulation are temporally coordinate in maize anthers. Plant J 7: 703–713.

Deluc L, Bogs J, Walker AR, Ferrier T, Decendit A, Merillon JM, et al. 2008. The transcription factor VvMYB5b contributes to the regulation of anthocyanin and proanthocyanidin biosynthesis in developing grape berries. Plant Physiol 147: 2041–2053.

Ferrante A, Hunter DA, Reid MS. 2004. Towards a molecular strategy for improving harvesting of olives (*Olea europaea L.*). Postharvest Biol Tech 31: 111–117.

Galla G, Barcaccia G, Ramina A, Collani S, Alagna F, Baldoni L, et al. 2009. Computational annotation of genes differentially expressed along olive fruit development. BMC Plant Biol 9: 128.

Hashim YZHY, Rowland IR, Mcglynn H, Servili M, Selvaggini R, Taticchi A, et al. 2008. Inhibitory effects of olive oil phenolics on invasion in human colon adenocarcinoma cells in vitro. Int J Cancer 122: 495–500.

Hichri I, Barrieu F, Bogs J, Kappel C, Delrot S, Lauvergeat V. 2011. Recent advances in the transcriptional regulation of the flavonoid biosynthetic pathway. J Exp Bot 62: 2465–2483.

Kamsteeg J, Vanbrederode J, Vannigtevecht G. 1980. Genetic and biochemical evidence that the hydroxylation pattern of the anthocyanin b-ring in silene-dioica is determined at the para-coumaroyl-coenzyme-a stage. Phytochemistry 19: 1459–1462.

Koes R, Werweij W, Quattrocchio F. 2010. Flavonoid: A colorful method for the regulation and evolution of biochemical pathways. Trends Plant Sci 10: 236–242.

Llorente-Cortes V, Estruch R, Mena MP, Ros E, Gonzalez MAM, Fito M, et al. 2010. Effect of Mediterranean diet on the expression of pro-atherogenic genes in a population at high cardiovascular risk. Atherosclerosis 208: 442–450.

Manning K. 1998. Isolation of a set of ripening-related genes from strawberry: Their identification and possible relationship to fruit quality traits. Planta 205: 622–631.

Moriguchi T, Kita M, Tomono Y, Endo-Inagaki T, Omura M. 2001. Gene expression in flavonoid biosynthesis: Correlation with flavonoid accumulation in developing citrus fruit. Physiol Plantarum 111: 66–74.

Olsen KM, Lea US, Slimestad R, Verheul M, Lillo C. 2008. Differential expression of four Arabidopsis PAL genes; PAL1 and PAL2 have functional specialization in abiotic environmental-triggered flavonoid synthesis. J Plant Physiol 165: 1491–1499.

Quattrocchio F, Wing JF, Leppen HTC, Mol JNM, Koes RE. 1993. Regulatory genes-controlling anthocyanin pigmentation are functionally conserved among plant-species and have distinct sets of target genes. Plant Cell 5: 1497–1512.

Renard CMGC, Dupont N, Guillermin P. 2007. Concentrations and characteristics of procyanidins and other phenolics in apples during fruit growth. Phytochemistry 68: 1128–1138.

Rewald B, Leuschner C, Wiesman Z, Ephrath JE. 2011. Influence of salinity on root hydraulic properties of three olive varieties. Plant Biosyst 145: 12–22.

Romani A, Mulinacci N, Pinelli P, Vincieri FF, Cimato A. 1999. Polyphenolic content in five Tuscany cultivars of Olea europaea L. J Agric Food Chem 47: 964–967.

Schijlen EGW, De vos CHR, Van tunen AJ, Bovy AG. 2004. Modification of flavonoid biosynthesis in crop plants. Phytochemistry 65: 2631–2648.

Sgromo C, Colao C, Reale L, Orlandi F, Rugini E, Fornaciari F, et al. 2010. Isolation and expression analysis of organelle genes involved in the development of olive flowers (Olea europaea L.). Plant Biosyst 144: 733–739.

Sparvoli F, Martine C, Scienza A, Gavazzi G, Tonelli C. 1994. Cloning and molecular analysis of structural genes involved in flavonoid and stilbene biosynthesis in grape (*Vitis Vinifera L.*). Plant Mol Biol 24: 743–755.

Takos AM, Jaffe FW, Jacob SR, Bogs J, Robinson SP, Walzer AR. 2006. Light-induced expression of a MYB gene regulates anthocyanin biosynthesis in red apples. Plant Physiol 142: 1216–1232.

Tognetti R, Morales-Sillero A, D'Andra R, Fernandez JE, Lavini A, Sebastiani L, et al. 2008. Deficit irrigation and fertigation practices in olive growing: convergences and divergences in two case studies. Plant Biosyst 142: 138–148.

Tripoli E, Giammarco M, Tabacchi G, Di Majo D, Giammarco SLA, La Guardia M. 2005. The phenolic compounds of olive oil: structure, biological activity and beneficial effects on human health. Nutr Res Rev 18: 98–112.

Tsuda T, Yamaguchi M, Honda C, Moriguchi T. 2004. Expression of anthocyanin biosynthesis genes in the epicarp of peach and nectarine fruit. J Amer Soc Hortic Sci 129: 857–862.

Van tunen AJ, Koes ER, Spelt CE, Van der krol AR, Stuitje AR, Mol JNM. 1988. Cloning of the calchone flavanone isomerase genes from petunia-hibrida coordinate light-regulated and differential expression of flavonoid genes. EMBO J 7: 1257–1264.

Vinha AF, Ferreres F, Silva BM, Valentao P, Goncalves A, Pereira JA, et al. 2005. Phenolic profiles of Portuguese olive fruits (*Olea europaea L.*): Influences of cultivar and geographical origin. Food Chem 89: 561–568.