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Isolation and functional characterization of a novel gene coding for flavonoid 3'-hydroxylase from globe artichoke

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Abstract

Globe artichoke (*Cynara cardunculus* L. var. *scolymus*) is rich in flavonoids which contribute to its health-promoting properties. With the aim of understanding the genetic control of flavonoid accumulation in artichoke, we isolated an artichoke full-length cDNA sequence encoding flavonoid 3'-hydroxylase (F3'H), a major enzyme of the flavonoid hydroxylation pattern. *In silico* studies confirmed that the deduced amino acid sequence of CcF3'H is highly similar to F3'Hs isolated from other *Asteraceae*. The Northern blot analysis demonstrated that *CcF3'H* was highly expressed in leaves and in specific parts of the heads. Its expression differed slightly among artichoke cultivars. The overexpression of *CcF3'H* in tobacco plants led to the accumulation of flavonoids and to an increase of flower colour intensity, thus identifying *CcF3'H* as promising candidate for genetic engineering. *CcF3'H* represents the first structural gene of the flavonoid biosynthesis isolated from *C. cardunculus*, and its characterization sheds light on the accumulation of flavonoids.

Additional key words: anthocyanins, *Cynara cardunculus*, expression analysis, flavonoid biosynthesis, transgenic plants.

Introduction

Flavonoids accumulate both constitutively and in response to environmental cues in flowers, fruits, leaves, and roots. They exert fundamental physiological roles as floral pigments and in plant defence and photoprotection (Holton and Cornish 1995, Treutter 2005, Zhang *et al*. 2012). Additionally, they contribute to the health-promoting value of the plants (Tsuda 2012). The anti-oxidant and radical scavenging capacities of flavonoids may be substantially modified by hydroxylation reactions (reviewed in Halbwirth 2010). Particularly, the hydroxylation of the B-ring influences colour, absorption of radiation, compound stability, and health- promoting effects (Forkmann 1991, Croft 1998, Halbwirth 2010). Flavonoid 3'-hydroxylase (F3'H) is responsible for hydroxylation of the B-ring at the 3' position, and has broad substrate specificity, catalysing the hydroxylation of flavanones, flavones, flavonols, and dihydroflavonols (Forkmann 1991, Winkel-Shirley 2001) (Fig. 1). Several F3'H-encoding genes have been isolated (Kitada *et al*. 2001, Seitz *et al.* 2006, Ishiguro *et al*. 2012) with the main objective of modifying flower colour in ornamentals (Nishihara and Nakatsuka 2011) but also for their role in the accumulation of health-promoting compounds (Bogs *et al*. 2006, Han *et al*. 2010).

Globe artichoke is an important component of the Mediterranean diet for its edible immature inflorescence (capitulum) and is considered a good source of natural antioxidants (Lattanzio *et al*. 2009). These properties are mainly related to flavonoids, such as the flavones luteolin and apigenin, and mono- and di-caffeoylquinic acids, such as chlorogenic acid (CGA) and cynarin (Wang *et al.* 2003, Shutz *et al.* 2004). Flavones have been detected in

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Abbreviations: ANS - anthocyanidin synthase; CGA - chlorogenic acid; DFR - dihydroflavonol 4-reductase; FHT - flavanone 3-hydroxylase; FLS - flavonol synthase; FNSII - flavone synthase II; F3'H - flavonoid 3'-hydroxylase; F3'5'H - flavonoid 3',5'-hydroxylase; *npt*II - neomycin phosphotransferase gene; ORF - open reading frame; RACE - rapid amplification of cDNA ends; RT-PCR - reverse transcription polymerase chain reaction; UTR - untranslated regions; WT - wild type.
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both artichoke leaves and heads (Schutz *et al*. 2004), with dihydroxylated flavones accumulating preferentially in intermediate bracts of the heads (Fratianni *et al.* 2007). Significant differences were also found among cultivars, including a local cultivar of Southern Italy, Tondo di Paestum, having a high content of beneficial flavones and mono-/di-caffeoylquinic acids (Fratianni *et al.* 2007). Among flavones, luteolin and to a lesser extent its glucosides have ability to inhibit cholesterol biosynthesis and lipid peroxidation (Brown and Rice-Evans 1998, Gebhardt 1998). Luteolin is synthesised through two possible reactions involving the cytochrome P_{450} enzymes F3'H and flavone synthase II (FNSII), with apigenin or eriodictyol as intermediates. F3'H also hydroxylates naringenin and dihydrokempferol thereby producing the cyanidin precursors eriodictyol and dihydroquercetin, respectively (Fig. 1). In this context, F3'H may have a major role in the accumulation of beneficial compounds, such as luteolin, and cyanidin-based pigments, the main anthocyanins in artichoke heads (Schutz *et al.* 2006).

Despite the long-known beneficial properties of globe artichoke phenylpropanoids, only a few genes of their metabolism have been isolated (Comino *et al.* 2007, 2009, De Paolis *et al.* 2008, Moglia *et al.* 2009, Sonnante *et al.* 2010). In order to contribute to the understanding of the mechanisms underlying the accumulation of flavonoids, we isolated a F3'H-encoding full-length cDNA sequence, *CcF3'H*, from globe artichoke and reported its expression pattern in various tissues and cultivars of artichoke. Moreover, we provide evidence that *CcF3'H* overexpression increases the accumulation of dihydroxylated flavones and other health-promoting compounds in transgenic tobacco plants.

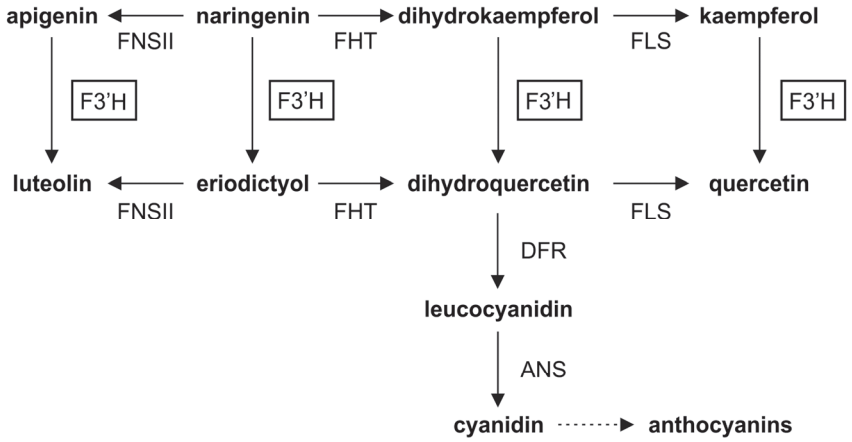

Fig. 1. The simplified representation of the part of the flavonoid biosynthetic pathway requiring flavonoid 3'-hydroxylase (F3'H). F3'H is highlighted by *rectangles*. ANS - anthocyanidin synthase, DFR - dihydroflavonol 4-reductase, FHT - flavanone 3-hydroxylase, FLS - flavonol synthase, FNSII - flavone synthase II (adapted from the *KEGG PATHWAY* database http://www.genome.jp/kegg/pathway.html).

Materials and methods

Plant: Globe artichoke (*Cynara cardunculus* L. var. *scolymus*) cv. Violet de Provence and the Italian landraces Bianco di Pertosa, Carciofo di Aquara, and Tondo di Paestum were grown in the field of the Agricultural Research Council, the Research Centre for Vegetable Crops (CRA-ORT), Pontecagnano, Italy, under standard management practices. Young leaves and heads were collected at commercial maturity from at least three different plants for each cultivar. Outer, intermediate, and inner bracts of the heads, receptacles, and leaves were separated and immediately frozen at -80 °C until further analyses.

Wild type (WT) and transgenic *Nicotiana tabacum* cv. Samsun shoots were grown *in vitro* on a Murashige and Skoog solid medium with 3 % (m/v) sucrose and with or without 100 mg dm^{-3} kanamycin at a temperature of 23 °C and a 16-h photoperiod with an irradiance of 40 μmol m^{-2} s^{-1}. After rooting, plantlets were transferred to soil and grown under the same temperature and photoperiod for three weeks. For determinations of flavones, flavonols, and anthocyanins in the control and transgenic tobacco lines, leaves and flowers at four different developmental stages were collected from three to five plants. Flower developmental stages were defined as follows: S0 stage, with unpigmented buds; S1 stage, with slightly pigmented buds; S2 stage, with buds just before anthesis; S3 stage, fully opened flowers. Plant materials were stored at -80°C until use.

Cloning full-length cDNA encoding F3'H in artichoke: Total RNA was isolated from 250 mg of intermediate bracts of the cv. Tondo di Paestum using the *Purelink Micro-to-Midi* total RNA purification system (*Invitrogen Life Technologies*, Carlsbad, USA) and treated with

DNAse I (*Invitrogen Life Technologies*) according to the manufacturer's instructions. The first-strand cDNA synthesis was carried out in a final volume of 20 mm^3 by mixing 1 μg of DNase-treated total RNA, 500 μg cm^{-3} oligo (dT)12-18 primers, and *Superscript II* reverse transcriptase according to the manufacturer's protocols (*Invitrogen Life Technologies*). The cDNA mixtures were used as templates for the PCR amplification of the target cDNA. Degenerate primers designed on the conserved EFNIGD and WAIARD regions of F3'H from other *Asteraceae* species were as follows: F-F3'H 5'-GWATTCAACATMGGHGACTT-3' and R-F3'H 5'-GGGTCDCGDGCTATGGCCC-3'. An expected fragment of about 570 bp for artichoke *F3'H* was amplified using the following cycling conditions: 94 °C for 2 min, 35 cycles (94 °C for 30 s, 50 °C for 1 min, 72 °C for 40 s), and a final extension at 72 °C for 10 min. The amplicon was then used to design primers for the amplification of the 5' and 3' ends using RACE-PCR (*GeneRacer*, *Invitrogen Life Technologies*) according to the supplier's protocol. The 5' end fragment of *CcF3'H* was amplified using a *GeneRacer*™ 5' primer and the reverse gene-specific primer FH-5'R: 5'-GGCGAA TAAGTTCAGCCATTGCCC-3'. The 3' RACE of *CcF3'H* was performed using a *GeneRacer*™ 3' primer and the forward gene-specific primer FH-3'R: 5'-GGG CAATGGCTGAACTTATTCGCC-3'. Final amplifications of the entire open reading frame (ORF) of *F3'H* was carried out from Tondo di Paestum intermediate bracts cDNA using *AccuPrime*™ *Pfx* DNA polymerase (*Invitrogen Life Technologies*) and gene-specific primers *Sac*I-FH 5'-CGAGCTCAACTCAAACAGAAACCC AAAACC-3' and *Xba*I-FH 5'-GCTCTAGATTTTTT TTTTCGTGATAAGAG-3' designed on the products of the 5' and 3' RACE for *CcF3'H*, and flanked by *Sac*I and *Xba*I restriction sites for subsequent cloning. Amplification conditions were: 94 °C for 2 min, 30 cycles (95 °C for 15 s, 53 °C for 30 s, 68 °C for 1 min and 40 s), and a final extension at 68 °C for 5 min. The PCR product was cloned into *pCR-BluntII-TOPO* vectors and sequenced using an *ABI3130* capillary sequencer (*Invitrogen Life Technologies*).

Bioinformatic analysis: Nucleotide sequences were translated into amino acid sequences using the *TRANSEQ* program (http://bioweb.pasteur.fr/seqanal/interfaces/transeq.html) and the deduced amino acid sequence subjected to a *BLAST* search *via* the GenBank database. Multiple sequence alignments and alignment design were performed using *BoxShade 3.2* (http://www.ch.embnet.org/software/BOX_form.html). Protein domains predictions were carried out on the website *EXPASY* (http://www.expasy.org).

A phylogenetic analysis was conducted *via* http://www.phylogeny.fr (Dereeper *et al.* 2008). A sequence alignment and an alignment refinement were performed using *Muscle v. 3.7* and *Gblocks 0.91b*, respectively, with default settings. The curated sequences were used for building a maximum likelihood phylogenetic tree using the *PhyML 3.0* algorithm (Guindon *et al.* 2010). The phylogenetic tree was statistically supported by the approximate likelihood-ratio test (aLRT).

Plasmid construction and stable tobacco transformation: The *Sac*I-*Xba*I fragment containing the full-length cDNA of *CcF3'H* was cloned into the *Sac*I-*Xba*I polylinker sites of the binary vector pKYLX71:35S^2 under the transcriptional control of the cauliflower mosaic virus 35S promoter with a double enhancer (Schardl *et al.* 1987). The correct fragment insertion was verified by sequencing. The resulting recombinant plasmid, pKY*CcF3'H*, was introduced into the *Agrobacterium tumefaciens* strain LBA4404 by the freeze-thaw method (Hofgen and Willmitzer 1988). Leaves from tissue cultured tobacco plantlets were infected and co-cultivated with *A. tumefaciens* cell suspensions harbouring either the pKY*CcF3'H* plasmid or the empty vector according to standard methods (Horsh *et al.* 1987). The stable insertion of the exogenous expression cassette was checked in several kanamycin-resistant plants by PCR amplification with primers for the neomycin phosphotransferase gene (*npt*II) (F-NptII 5'-AGGCTATTCGGCTATGACTGGGCA-3'; R-NptII 5'-TCAGAAGAACTCGTCAAGAAGGCG-3').

Expression analysis: For Northern blot analysis, total RNA was extracted from leaves and heads by the guanidiniumisothiocyanate method (Chomeczynski and Sacchi 1987). Equal amounts of RNA (20 μg) were separated and transferred onto *Hybond-*N^+ membranes (*GE Healthcare, Amersham Biosciences*, Buckinghamshire, UK) according to the manufacturer's instructions. Filters were hybridized with **[**α^{32}**P]**dCTP labelled cDNA fragments of the artichoke *F3'H* (0.5 kb) gene. RNA gel blot hybridisation was carried out under high stringency conditions with 40 % (m/v) formamide at 42 °C. After the hybridisation, the membranes were washed twice in 2× saline-sodium citrate buffer (SSC) and 0.1 % (m/v) sodiumdodecylsulphate (SDS) at 65 °C for 15 min and twice in 0.1× SSC and 0.1 % SDS at 65 °C for 15 min. Band detection was performed using a laser scanner *Typhoon 9200* (*GE Healthcare*).

For semi-quantitative RT-PCR analysis of the *CcF3'H* transgene in the transformed tobacco plants, 1 μg of total RNA from leaves of two *CcF3'H* transformants, plants transformed with the empty pKYLX71:35S^2 vector, and WT plants were retro-transcribed using *SuperScript II* reverse transcriptase (*Invitrogen Life Technologies*). RT-PCR amplifications were performed, at least three times from different plants, using a specific primer pair designed on the artichoke *F3'H* gene sequence (FH-3'R 5'-GGGCAATGG CTGAACTTATTCGCC-3') and on the polylinker region of the pKYLX71:35S^2 vector (R-CH11 5'-GAAACT GATGCATTGAACTTG-3'). As semi-quantitative control, tobacco *actin* was amplified with specific primers (F-act 5'-AGGGTTTGCTGGAGATGATG-3';

R-act 5'-CGGGTTAAGAGGTGCTTCAG-3'). A reaction mixture contained 1× PCR buffer, 0.2 mM dNTPs, 1.5 mM $MgCl_2$, 1.5 units of *Taq DNA* polymerase (*Invitrogen Life Technologies*), 500 nM each primer, and 1 mm^3 of a cDNA template in a final volume of 50 mm^3. The amplification program started with a step at 95 °C for 5 min followed by 35 cycles (95 °C for 30 s, 50 °C for 1 min, and 72 °C for 45 s) and a final extension at 72 °C for 10 min. Amplified products were separated and visualized on a 1 % (m/v) ethidium bromide (EtBr) agarose gel.

Quantification of flavonoids in transgenic tobacco: For flavonoid analysis of the control and two selected *CcF3'H* over-expressing lines, frozen leaves and flowers at four developmental stages from three to five tobacco plants were ground in liquid nitrogen, extracted in 80 % (v/v) aqueous methanol solution and then sonicated three times for 1 min. Soluble phenolics were separated by centrifuging (*Heraeus Biofuge Fresco*, Waltham, USA) at 16 000 *g* for 5 min and stored at -20 °C until use. HPLC analysis was performed as follows: 20 mm^3 of each extract was analysed by reverse phase (RP)-HPLC using a *Gold System* chromatograph equipped with an UV detector (*Beckman*, Brea, USA) and a *KR 100-5C18 Khromasyl* column at a flow rate of 0.7 cm^3 min^{-1}. The mobile phase included HPLC-grade water [containing 0.01 % (v/v) trifluoroacetic acid; solvent A] and 95 % (v/v) acetonitrile (containing 0.01 % trifluoroacetic acid; solvent B) in the following gradient system: initial 5 % A, a linear gradient to 50 % B in 35 min, a linear gradient to 100 % B in 3 min, and coming back to the initial step of 5 % A. Chromatograms were recorded at λ = 280 nm. Standards for apigenin and luteolin were obtained from *Extrasynthese* (Genay, France), whereas for kaempferol, naringenin, and quercetin from *Sigma-Aldrich* (Milano, Italy).

To measure total anthocyanins in flowers and leaves of WT and the same two independent tobacco lines used for analysis of apigenin and luteolin, anthocyanins were extracted from three to five plants with 1 cm^3 of 1 % (m/v) HCl in methanol in the dark at 4 °C overnight. Extracts were centrifuged at 16 000 *g* for 5 min and supernatants were spectrophotometrically measured at 530 and 657 nm, the latter wavelength corresponding to the chlorophyll absorbance. The anthocyanin content was calculated as A_{530} minus A_{657} and expressed per gram of fresh mass (f.m.). Fifteen treatments obtained from the factorial combination of three genotypes (the WT and the two transgenic lines), and five tissues (flower developmental stages S0, S1, S2, and S3, and leaves) were compared. A split plot design was arranged with $n = 3$ to 5. Statistical data processing was performed with *ANOVA*, referring to $P \leq 0.05$, and the Duncan's test was used for means separation.

Results

In order to isolate and characterise the gene encoding flavonoid 3'-hydroxylase (F3'H) from the non-model plant, globe artichoke, amino acid sequences for F3'H enzymes of *Asteraceae* species were retrieved from databases and aligned. Degenerated primers designed on conserved regions (Fig. 2) amplified an incomplete F3'H-encoding sequence which was extended towards both 3'- and 5'-ends by RACE-PCR from intermediate bracts of the globe artichoke cv. Tondo di Paestum.

The F3'H-encoding full-length cDNA, designated *CcF3'H* (GenBank acc. No. HM153534), was 1 619 bp in length including the 5' and 3' untranslated regions (UTRs) and contained a putative open reading frame (ORF) of 1 539 bp encoding 512 amino acid residues. The deduced amino acid sequence of *CcF3'H* exhibited the best matches of identity mainly with F3'H proteins isolated from *Centaurea cyanus* (90 %), *Cichorium intybus* (86 %), *Echinops bannaticus* (85 %), and *Gerbera hybrida* (83 %), and shared a high identity also with F3'H from other than *Asteraceae* species, such as *Torenia hybrida* (73 %) and *Gentiana triflora* (72 %). *CcF3'H* also showed a relatively high identity with flavonoid 3',5'-hydroxylase (F3'5'H), another essential enzyme involved in hydroxylation of the flavonoid B-ring, from *Osteospermum hybrida* (76 %), *Callistephus chinensis* (74 %), and *Pericallis cruenta* (73 %).

The identification of conserved domains and motif characterization allowed the assignment of *CcF3'H* to the cytochrome P_{450} family (Fig. 2). *CcF3'H* showed also the consensus sequence 'GGEK' at amino acid positions 421 - 424 which is highly conserved in F3'H but not in F3'5'H sequences (Brugliera *et al.* 1999).

The phylogenetic analysis of translated sequences shows that *CcF3'H* lies within the sub-cluster of *Asteraceae* F3'H sequences (Fig. 3) in which the highly identical *Asteraceae* specific F3'5'Hs form a distinct group. Evidence that the artichoke deduced amino acid sequence clusters mainly with F3'H of other *Asteraceae*, is separated from *Asteraceae* F3'5'Hs, and contains all the characteristic motifs conserved in P_{450} monooxygenases including the consensus sequence GGEK, indicates that the cDNA clone isolated in this study encoded a globe artichoke F3'H enzyme.

The transcription of the newly isolated artichoke gene, *CcF3'H,* was examined in specific parts of the head, namely outer, intermediate, and inner bracts, in receptacles, and in young leaves collected at commercial maturity of the head of the cv. Tondo di Paestum. Northern blot analysis shows that *CcF3'H* was slightly detectable in the receptacles, whereas it had a higher transcription in all the other tissues (Fig. 4*A*). The expression pattern of *CcF3'H* was investigated also in intermediate bracts and young leaves of four artichoke cultivars (Carciofo di Aquara, Tondo di Paestum, Bianco

di Pertosa, and Violet de Provence). Northern blot analysis indicates that *CcF3'H* was transcribed at similar levels in all the tested cultivars and was consistently more expressed in leaves than in intermediate bracts. Only in the cv. Tondo di Paestum, the intermediate bracts showed a higher *CcF3'H* transcription, comparable to that found in the leaves (Fig. 4*B*).

Demonstration of functional activity and further characterisation of *CcF3'H* was achieved through the stable transformation of *N. tabacum* cv. Samsun with an *A. tumefaciens* recombinant strain carrying *CcF3'H* under the CaMV35S[2] promoter into a binary vector (pKY*CcF3'H*). Two kanamycin-resistant independent T2 transformants (#9 and #14), selected after PCR amplification with primer pairs for the kanamycin resistance gene *npt*II (data not shown), were self-pollinated and used for further analyses.

The expression of *CcF3'H* was evaluated in *in vivo*

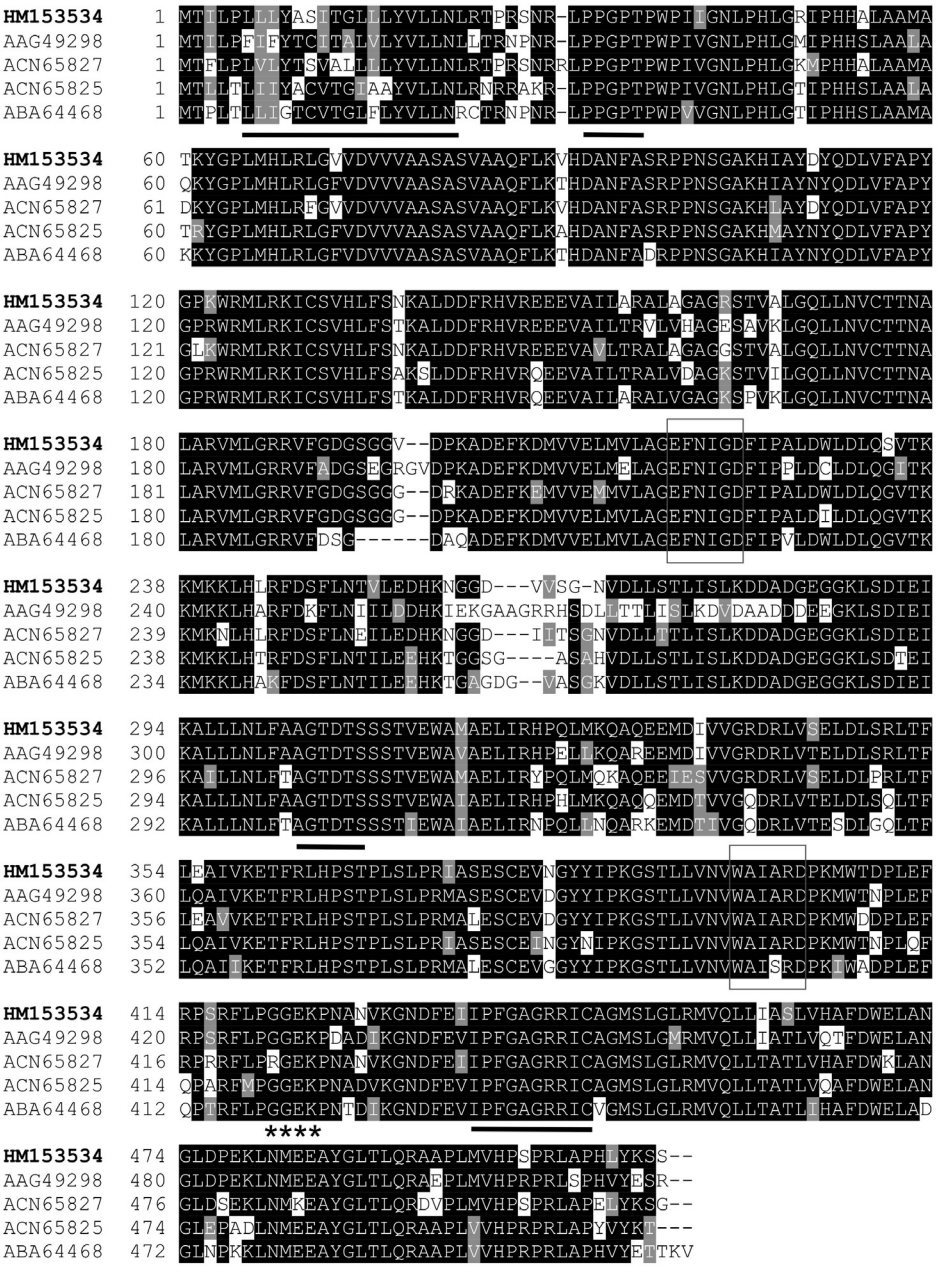

Fig. 2. The alignment of the *CcF3'H* deduced amino acid sequence with corresponding selected sequences from *Asteraceae*. Acc. Nos. in the GenBank database are: *Cynara cardunculus* var. *scolymus* [(HM153534) this study, marked in *bold*], *Callistephus chinensis* (AAG49298), *Centaurea cyanus* (ACN65827), *Cychorium intybus* (ACN65825), *Gerbera hybrida* (ABA64468). Positions of degenerated primers designed on conserved structural motifs are indicated by *open boxes*. The membrane anchored hydrophobic region, the proline rich region (PPxP), the oxygen binding pocket [AGTDT(T/S)], and the heme-binding region (FGxGRRxCxG) are *underlined*. The 'GGEK' sequence (position 421-424), characteristic of F3'H sequences, is indicated by *asterisks*.

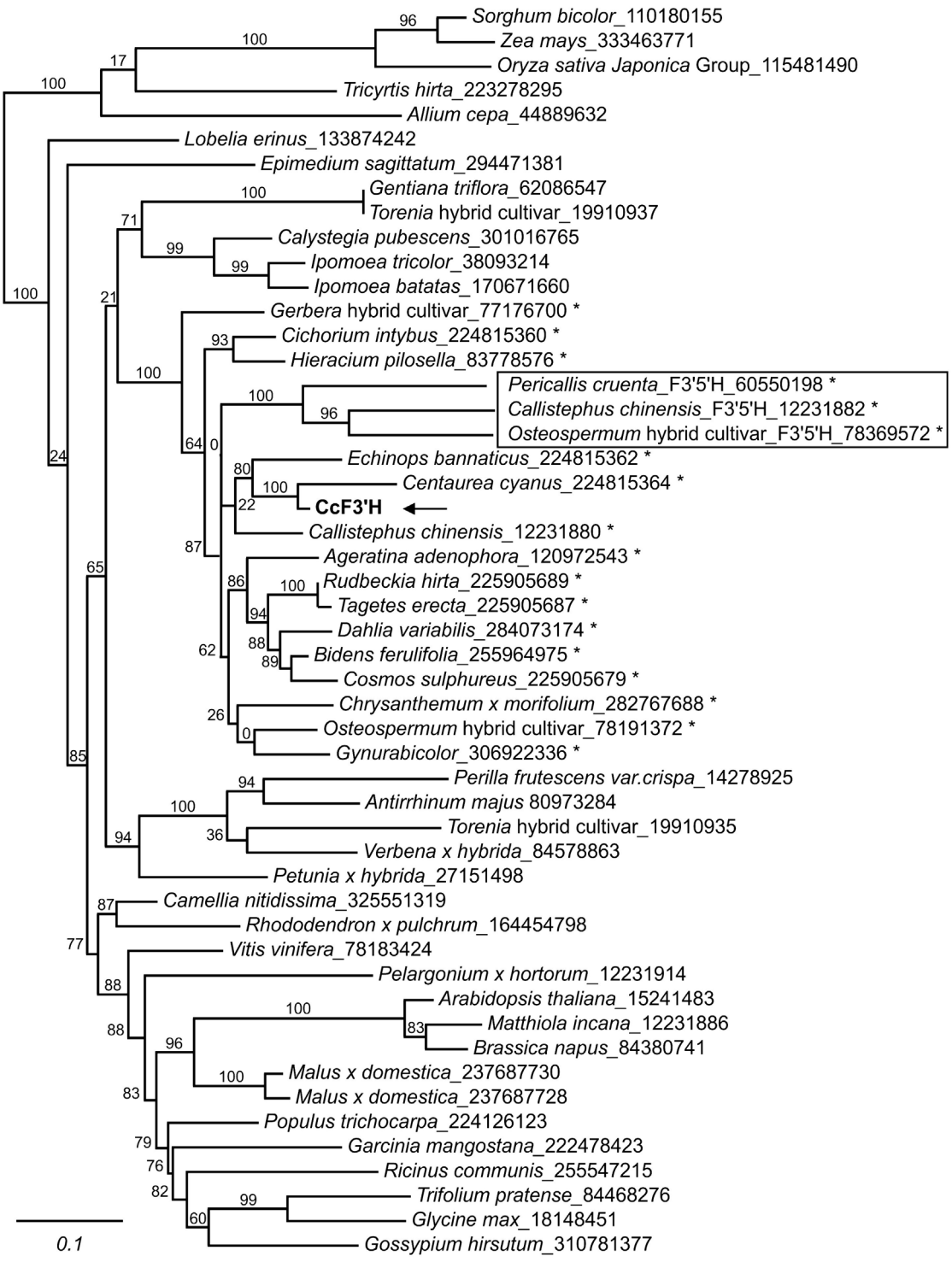

Fig. 3. The phylogenetic analysis of F3'H on the basis of the alignment of amino acid sequences from different plant species. The phylogenetic tree was produced by the maximum likelihood method using http://www.phylogeny.fr. Numbers next to the nodes indicate the approximate likelihood-ratio test (aLRT) branch support. Amino acid sequences are identified by species and GI number, with the *Asteraceae* being labelled by the *asterisk*. The artichoke sequence isolated in this study is indicated by the *arrow*. F3'5'H sequences showing similarity with the deduced amino acid sequence of *CcF3'H*, all belonging to *Asteraceae* species, are also included in the alignment and highlighted by the *open box*.

grown plants of lines #9 and #14 by semiquantitative RT-PCR using gene-specific primers. WT and empty vector transformed plants were also analysed as controls. A *CcF3'H* transcript accumulation was detected in both #9 and #14 lines, whereas, as expected, no amplification was obtained from the WT and empty vector transformed plants (Fig. 5*A*).

The overexpression of *CcF3'H* in tobacco plants did not cause any apparent phenotypic effect under normal growth conditions except for a change in the colour of flowers which were of a deeper pink in the transgenic plants (Fig. 5*B*).

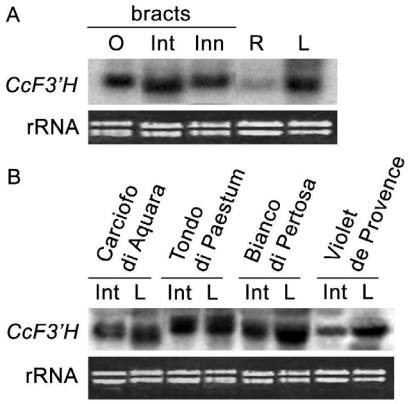

Fig. 4. The expression analysis of the artichoke gene *CcF3'H* in the head and leaf of different artichoke cultivars. Total RNA was isolated from outer (O), intermediate (Int) and inner (Inn) bracts, receptacle (R), and leaves (L) of the cv Tondo di Paestum (*A*) and from intermediate bracts (Int) or leaves (L) of four artichoke cultivars (*B*). Filters were hybridized with a [α^{32}P]dCTP labelled cDNA fragment (0.5 kb) of *CcF3'H* (GenBank acc. No. HM153534). Ethidium bromide stained ribosomal RNA bands are shown as loading control.

Amounts of relevant flavonoids hydroxylated by F3'H, *i.e.*, luteolin with its precursor apigenin and quercetin with its precursor kaempferol (Fig. 1) were determined by HPLC analysis in flowers at four different developmental stages and in leaves of the WT and transgenic tobacco lines #9 and #14 (Fig. 6 and Table 1). In the WT tobacco, apigenin was detected as the major flavone only in developing flowers, mainly at the S1stage [508.0 μg g^{-1}(f.m.)], whereas luteolin was present only at the S2 stage [1.2 μg g^{-1}(f.m.)]. In the transgenic line #9,

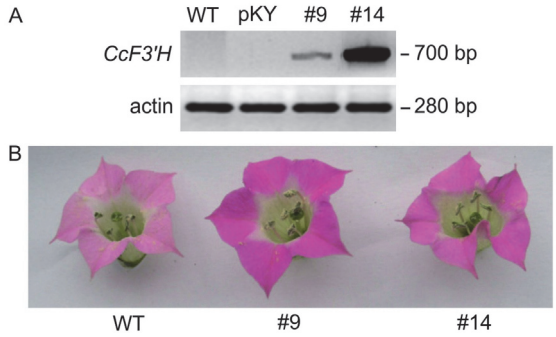

Fig. 5. The gene expression analysis of *CcF3'H*, and changes in the flower colour in transgenic tobacco plants. *A* - the RT-PCR analysis of the transcript accumulation of *CcF3'H* (the *upper panel*) and of the housekeeping tobacco *actin* gene (the *lower panel*) in an untransformed control (WT), in a tobacco line transformed with the empty vector (pKY), and in *CcF3'H*-overexpressing T2 lines (#9 and #14). Results are representative of at least three RT-PCR amplifications from different plants. *B* - Fully opened flowers of WT and *CcF3'H*-overexpressing transgenic tobacco plants (lines #9 and #14).

Fig. 6. Tobacco flower developmental stages (S0, S1, S2, and S3) and leaves (L) assessed for the content of flavonoids.

Table 1. The amounts of apigenin, luteolin and quercetin [μg g^{-1}(f. m.)] in flowers at four developmental stages (S0, S1, S2, and S3) and in leaves (L) determined by HPLC analysis in the untransformed control (WT) and *CcF3'H*-overexpressing T2 tobacco lines (#9 and #14). Means ± SD, n = 3 to 5. Values indicated by the same letter are not significantly different (P > 0.05) according to the Duncan's test.

Flavonoids	Lines	S0	S1	S2	S3	L
Apigenin	WT	51.56 ± 9.50de	507.97 ± 71.1a	72.86 ± 13.3d	1.50 ± 0.40f	0.00 ± 0.00f
	#9	308.90 ± 47.2b	27.65 ± 7.87ef	174.64 ± 24.4c	57.73 ± 12.18de	9.76 ± 1.46f
	#14	57.08 ± 7.99de	9.11 ± 3.27f	60.27 ± 10.4de	24.86 ± 5.48ef	2.87 ± 0.50f
Luteolin	WT	0.00 ± 0.00c	0.00 ± 0.00c	1.22 ± 0.17b	0.00 ± 0.00c	0.06 ± 0.01c
	#9	0.00 ± 0.00c	6.94 ± 1.16a	1.20 ± 0.30b	0.00 ± 0.00c	0.00 ± 0.00c
	#14	0.00 ± 0.00c	0.00 ± 0.00c	0.00 ± 0.00c	0.39 ± 0.07c	1.20 ± 0.30b
Quercetin	WT	0.00 ± 0.00e	0.00 ± 0.00e	0.21 ± 0.04e	0.00 ± 0.00e	0.00 ± 0.00e
	#9	6.43 ± 0.80a	6.15 ± 0.88a	1.28 ± 0.19d	1.30 ± 0.22d	0.00 ± 0.00e
	#14	3.39 ± 0.52b	6.18 ± 0.70a	3.18 ± 0.35b	2.48 ± 0.40c	0.28 ± 0.07e

Table 2. Anthocyanin accumulation in flowers at four developmental stages (S0, S1, S2, and S3) and in leaves (L) of an untransformed control (WT) and *CcF3'H*-transgenic T2 tobacco lines (#9 and #14). The anthocyanin content was determined spectrophotometrically and expressed as $(A_{530}-A_{657})\ g^{-1}$(f. m.). Means ± SE, n = 3 to 5. Values indicated by the same letter are not significantly different ($P > 0.05$) according to the Duncan's test.

Tobacco line	S0	S1	S2	S3	L
WT	0.04 ± 0.00a	0.47 ± 0.24a	0.68 ± 0.12b	1.32 ± 0.14c	0.17 ± 0.07a
#9	0.02 ± 0.01a	0.13 ± 0.02a	0.85 ± 0.21b	4.23 ± 0.74a	0.02 ± 0.01a
#14	0.27 ± 0.03a	0.55 ± 0.04a	1.87 ± 0.10a	2.81 ± 0.98b	0.02 ± 0.00a

luteolin was not detected at an early stage of flower development (S0), showed a peak of accumulation at the S1 stage [6.9 μg g^{-1}(f.m.]), and decreased later on (Table 1). In the same transgenic line, an opposite trend was shown by apigenin which was present at the S0 stage and decreased at later stages, particularly at the S1, when the luteolin content peaked. The transgenic line #14 showed a lower accumulation of both flavones compared to the WT and the transgenic line #9 (Table 1). Leaves did not show any detectable amount of apigenin both in the WT and in the transgenic plants, whereas luteolin was slightly present only in the transgenic line #14 (Table 1).

The flavonol quercetin accumulated only in the two transgenic lines, mainly at early stages of flower development (S0 and S1) and decreased afterwards (S2 and S3) (Table 1), whereas kaempferol and naringenin were never detected. Naringenin and both flavonols were not found in leaves of the WT and transgenic plants.

The total anthocyanin content was measured in flowers at four developmental stages and in leaves of the WT plants and the transgenic tobacco lines (Fig. 6 and Table 2). Anthocyanins accumulated more in the transgenic than in the WT flowers ($P \leq 0.05$). A significant interaction between the genotype and tissue indicated a higher anthocyanin accumulation in the transgenic line #14 already at stage S2. At stage S3, corresponding to fully opened and pigmented flowers, the highest anthocyanin content was detected in the transgenic line #9 (Table 2). Conversely, very small amounts of anthocyanins were detected in leaves regardless of the genotype.

Discussion

The well-known beneficial effects of globe artichoke on human health are mainly attributed to its elevated content of phenolic compounds, and especially in hydroxy-cinnamic acids (CGA and cynarin) and flavones (apigenin and luteolin) (Lattanzio *et al.* 2009). Besides their interest for human health, flavones have multiple functions in plants, being important UV protectants (Harborne and Williams 2000) and essential contributors to nodulation (Zhang *et al.* 2007) and plant defence (Soriano *et al.* 2004). Furthermore, flavones and flavonols act as co-pigments contributing to an intensification of flower colour (Martens and Mithofer 2005). In this work, we have addressed the mechanisms underlying the accumulation of flavonoids in artichoke through the isolation of *CcF3'H* involved in the hydroxylation reactions leading to the accumulation of luteolin, the main artichoke flavone, and other beneficial compounds.

The complete cDNA sequence of *CcF3'H* was obtained from intermediate bracts of the edible artichoke inflorescence using a PCR-based cloning strategy with degenerate primers, followed by 3' and 5' RACE. As expected, the sequence comparison shows that *CcF3'H* shared the highest identities (80 - 90 %) with the corresponding sequences of F3'H from other *Asteraceae*, whereas the identity was lower with other species. The *CcF3'H* deduced sequence had also a significant identity (up to 70 %) with *Asteraceae* specific F3'5'Hs which in fact were proposed to have arisen from F3'H in this family (Seitz *et al.* 2006). However, the finding that the *CcF3'H*-encoded sequence contains the F3'H-exclusive sequence "GGEK", which was never detected in the highly similar P_{450} enzymes F3'5'H (Brugliera *et al.* 1999) and is conceivably involved in the functional difference between the two enzymes, strongly indicates that *CcF3'H* encodes a F3'H enzyme. This conclusion was confirmed by the phylogenetic analysis showing that *CcF3'H* translated sequence is closely related to F3'H from other *Asteraceae* and does not group together with *Asteraceae* specific F3'5'Hs. The sequence analysis identified the characteristic cytochrome P_{450} motifs. The deduced sequence displays the proline-rich "hinge" region essential for an optimal orientation of the enzyme (Yamazaki *et al.* 1993) along with the oxygen binding pocket and the heme domain which are required for oxygen and heme iron binding, respectively. Furthermore, it possesses the N-terminal hydrophobic region important for a proper orientation and targeting the protein to the microsomal membrane (Sato *et al.* 1990, Murakami *et al.* 1994) which is essential for the assembly of macromolecular complexes (metabolons) involved in metabolic channelling, enhancing the specificity, and efficiency of biochemical pathways (Winkel 2004).

An in-depth characterisation of the transcriptional patterns of *CcF3'H* was carried out in the cv. Tondo di

Paestum. This analysis shows that *CcF3'H* was highly transcribed in leaves and in all parts of the head, except for the receptacle. Many studies have reported the transcriptional analysis of F3'H-encoding genes in different species, mostly restricted to different stages of flower or fruit development (Ueyama *et al.* 2002, Nakatsuka *et al.* 2005, Bogs *et al.* 2006). A few studies have compared *F3'H* expression in other plant organs and tissues including leaves. In several species, a high expression of *F3'H* was found in leaves similarly to our findings (Schoenbohm *et al.* 2000, Kitada *et al.* 2001, Schlangen *et al.* 2010), whereas it was generally lower in stems or roots (Brugliera *et al.* 1999, Schoenbohm *et al.* 2000, Nakatsuka *et al.* 2005). However, to our knowledge, this is the first report concerning the head, an organ peculiar to artichoke, which was only investigated for phenylalanine ammonia-lyase and hydroxycinnamoyl-CoA quinate transferase gene expressions (De Paolis *et al.* 2008, Sonnante *et al.* 2010). Further transcriptional profiling *CcF3'H* in different tissues and cultivars of globe artichoke demonstrated that its expression is generally higher in leaves than in intermediate bracts, whereas no major differences were detected between cultivars.

Individual health-promoting compounds were recently demonstrated to accumulate in well-defined parts of the artichoke head and in specific genotypes (Fratianni *et al.* 2007, Lombardo *et al.* 2010, Sonnante *et al.* 2010). In the same artichoke cultivars used in this study, luteolin accumulated in intermediate bracts and was almost undetectable in young leaves (Fratianni *et al.* 2007), opposite to the expression of *CcF3'H* found in the same tissues (Fig. 4*B*). As for differences between cultivars, the highest luteolin accumulation was demonstrated in intermediate bracts of Violet de Provence (up to 5.7 μmol g^{-1}; Fratianni *et al.* 2007), which showed a low *CcF3'H* expression (Fig. 4*B*). Those results suggest that the luteolin accumulation did not correlate with the *CcF3'H* transcription rate in globe artichoke. Similarly, *F3'H* expression does not correlate with anthocyanin accumulation in chrysanthemum flowers (Chen *et al*. 2012). A possible explanation for this lack of correlation between the expression of genes and the accumulation of their products may be the unavailability of the relevant substrates in specific tissues. Moreover, any deficiency in the coordinate regulation of the enzymes of the metabolons required for flavonoid biosynthesis may result in a reduced efficiency of the pathway and an impaired accumulation of specific metabolites (Winkel 2004, Crosby *et al.* 2011).

The *CcF3'H* ability to catalyse the introduction of hydroxyl groups in the B-ring, leading to the synthesis of luteolin, quercetin, and precursors of cyanidin-based pigments, was verified *in vivo* through its transformation in tobacco, a species which does not accumulate flavones in fully opened flowers (Nakatsuka *et al.* 2006). In the *CcF3'H* expressing tobacco line #9, an increase in luteolin was observed compared to the WT, particularly in the S1 stage of flower development, and it was accompanied by a clear decrease in the apigenin content at the same stage which is consistent with a possible role of apigenin as luteolin precursor in globe artichoke. However, in the independent transgenic line #14, the similar negative peak of apigenin accumulation at the S1 stage was not accompanied by an increase in luteolin. Metabolic engineering through the overexpression of biosynthetic and modifying enzymes in transgenic plants has often led to unexpected results demonstrating the complex regulation of plant secondary metabolism. F3'H, together with other enzymes of the pathway, is organised in a membrane-anchored metabolon which increases specificity and efficiency of the pathway (Winkel 2004). Affinity of the gene product from another species with enzymes of the metabolon, the need for a tightly co-regulated expression of the relevant genes, and/or unavailability of intermediates could explain the absence of correlation between the gene expression and accumulation of reaction products of the encoded enzyme. However, the possibility that, unlike the low *CcF3'H* expression in line #9, the high transcriptional activity of *CcF3'H* in line #14 could have exceeded the threshold level that triggers post-transcriptional silencing mechanisms (Wassenegger and Pélissier 1998) could not be ruled out. The residual gene expression could have been sufficient to produce enough F3'H enzyme in the transgenic line #14 to drive the accumulation of quercetin and anthocyanin precursors (see below).

The constitutive *CcF3'H* overexpression also resulted in an increased production of flavonols and anthocyanins. Similarly to what reported for several species (Winkel-Shirley 2002, Noda *et al.* 2004) the content of the flavonol quercetin in the two transgenic tobacco lines increased prior to the anthocyanin accumulation during floral development (S1) and declined when anthocyanins began to accumulate (S3) (Figs. 6 and 7), possibly due to competition between enzymes involved in the biosynthetic pathways for the common substrate dihydroquercetin (Holton *et al.* 1993). The stage-specific synthesis of flavonols and anthocyanins is ascribed to developmentally and temporally coordinated transcription of structural genes of the flavonoid pathway during flower pigmentation (Noda *et al.* 2004). Our results indicate that this tight regulation may also extend to transgenic plants constitutively overexpressing *CcF3'H*. Consistently with the higher anthocyanin production, flowers of the two *CcF3'H*-overexpressing tobacco lines showed a deeper pink colour, particularly in the S3 stage. These results are in accordance with studies demonstrating the ability of F3'Hs isolated from several plant species to enhance colour intensity of petals of transgenic ornamental and model plants, mainly by the production of 3'-hydroxylated (cyanidin-type) anthocyanins (Ueyama *et al.* 2002, Nakatsuka *et al.* 2006, Han *et al.* 2010).

Controversial data were reported on the efficiency of the same F3'Hs in catalysing flavone hydroxylation in transgenic plants (Ueyama *et al.* 2002, Nakatsuka *et al.* 2006), although different specificities of F3'Hs from

different species have been clearly demonstrated (Ueyama *et al.* 2002 and references therein). Tobacco plants transformed with *GtF3'H* from *Gentiana triflora* show an enhanced accumulation of anthocyanins but not of flavones in flowers (Nakatsuka *et al.* 2006). Conversely, F3'H from *Torenia hybrida* is able to increase cyanidin-type anthocyanins, resulting in a redder flower colour, and is also associated with a luteolin accumulation, similarly to *CcF3'H* (Ueyama *et al.* 2002). Our results demonstrate that the expression of the artichoke *CcF3'H* gene enhanced both dihydroxylated flavone and flavonol as well as anthocyanin accumulations.

In this paper, we report the isolation and characterisation of the *CcF3'H* gene from globe artichoke as a first contribution to the understanding of the genetic control of flavonoid modification patterns leading to an accumulation of beneficial compounds in this non-model species. Overexpression in transgenic tobacco demonstrated the functional activity of *CcF3'H* in increasing dihydroxylated flavone and flavonol, and anthocyanin accumulation *in vivo*. Therefore, *CcF3'H* may be a good candidate for biotechnological applications aimed at obtaining new flower colours or at increasing the production of compounds important both for the physiology of the plant and for the promotion of human health.

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