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Isolation and mapping of a C3'H gene (CYP98A49) from globe artichoke, and its expression upon UV-C stress

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Abstract

Globe artichoke represents a natural source of phenolic compounds with dicaffeoylquinic acids along with its biosynthetic precursor chlorogenic acid (5-caffeoylquinic acid) as the predominant molecules. We report the isolation and characterization of a full-length cDNA and promoter of a globe artichoke *p*-coumaroyl ester 3'-hydroxylase (CYP98A49), which is involved in both chlorogenic acid and lignin biosynthesis. Phylogenetic analyses demonstrated that this gene belongs to the CYP98 family. CYP98A49 was also heterologously expressed in yeast, in order to perform an enzymatic assay with *p*-coumaroylshikimate and *p*-coumaroylquinic acid as substrates. Real Time quantitative PCR analysis revealed that CYP98A49 expression is induced upon exposure to UV-C radiation. A single nucleotide polymorphism in the CYP98A49 gene sequence of two globe artichoke varieties used for genetic mapping allowed the localization of this gene to linkage group 10 within the previously developed maps.

Keywords: globe artichoke, C₃H, genetic mapping, UV-C radiation

Introduction

Globe artichoke ($2n=2x=34$, *Cynara cardunculus* L. var. *scolymus*) is a perennial, allogamous, primarily vegetatively propagated vegetable, native to the Mediterranean basin. Its cultivation makes a considerable contribution to the agricultural economy of southern Europe, with Italy being the major world producer (about 470 Mt per year). Globe artichoke is used mostly for human food, although the polyphenolic content of its leaves is known to have therapeutic properties. The predominant phenolics present in the leaf are the dicaffeoylquinic acids and chlorogenic acid (5-caffeoylquinic acid) (Lattanzio et al. 1994; Wang et al. 2003). These metabolites possess antioxidative, hepatoprotective, diuretic and choleric activity (Adzet et al. 1987; Gebhardt 1997; Brown and Rice-Evans 1998). Leaf extracts have also been reported to inhibit cholesterol biosynthesis, to contribute to the prevention of arteriosclerosis and other cardiovascular diseases (Gebhardt 1998), and may inhibit HIV integrase, a key enzyme in viral replication and insertion into host DNA (Slanina et al. 2001).

The reconstruction of the phenylpropanoid pathway and the elucidation of the biological functions of secondary metabolites is an area of active research (Douglas 1996). Phenylpropanoid compounds, which include flavonoids, lignin, coumarins and many small phenolic compounds, contribute to a multiplicity of plant functions, such as the strengthening of the cell wall, pigmentation of the flower, defence against pathogens and cell signalling (Boudet 2007).

To date few studies on phenylpropanoid biosynthetic pathway have been performed in globe artichoke. Three sequences with high similarity to PAL (phenylalanine ammonia-lyase), the first enzyme involved in the phenylpropanoid pathway, have been isolated (De Paolis et al. 2008). Moreover, gene sequences encoding hydroxycinnamoyltransferase (HCT and HQT), involved in the synthesis of chlorogenic acid, have been recently identified (Comino et al. 2007; Comino et al. personal communication). New insights are thus required for identifying other genes involved in caffeoylquinic acid synthesis and elucidating the causal relationships between the various enzymes.

The 3-hydroxylation of the phenylpropanoid ring is essential not only for the synthesis of lignin (Franke et al. 2002; Abdulrazzak et al. 2007; Chen and Dixon 2007), but is also an important step in the synthesis of chlorogenic acid (Schoch et al. 2006, Mahesh et al. 2007). The CYP98 family of plant cytochromes P450 have been designated as the family of enzyme that performs the *meta*-hydroxylation step in the phenylpropanoid pathway (Fig. 1). This *meta*-hydroxylation is not catalyzed on free *p*-coumaric acid, but on its conjugates with shikimic, quinic or phenyllactic acids (Schoch et al. 2006). The protein and encoding genes in this family are also referred to as C3'H.

The phenylpropanoid pathway is activated upon exposure to abiotic and biotic stresses, such as wounding, UV irradiation and pathogen attack (Dixon and Paiva 1995; Treutter 2005). The involvement in the stress response of chlorogenic acid has been reported, with its concentration increasing in lettuce upon wounding, and in tobacco upon both UV-B irradiation and insect feeding (Cantos et al. 2001; Izaguirre et al. 2007). In globe artichoke, we have recently shown that exposure to UV-C consistently increases the level of major dicaffeoylquinic acid isomer, while the exogenous application of either methyl jasmonate or salicylic acid has no such effect (Moglia et al. 2008). Furthermore we previously generated the first genetic map of globe artichoke, based on a two-way pseudo-testcross strategy (Lanteri et al. 2006). An F₁ population was created by crossing 'Romanesco clone C3' (a late-maturing, non-spiny type) with 'Spinoso di Palermo' (an early-maturing spiny type), and the progeny was genotyped using a number of marker types, such as AFLP, S-SAP, SSR and M-AFLP (Lanteri et al. 2006).

The main objectives of this paper are (a) the cloning of a C3'H gene and its promoter from globe artichoke, (b) the study of its transcriptional regulation in response to UV-C irradiation and (c) the definition of the gene's localization in a previously developed map in relation to other DNA-based markers. The *in vitro* enzymatic assay of C3'H was also performed.

Materials and Methods

Plant material, DNA and RNA extraction

Seeds of globe artichoke ('Concerto', Nunhems) were germinated on a 15 cm Petri dish on two layers of wetted filter paper, and after two weeks transplanted into soil-filled 10 cm pots in a glasshouse held at 22-24°C for ten weeks. DNA was extracted from leaves, following Lanteri et al. (2001). Total RNA was extracted from approximately 100 mg fresh leaf using the Trizol reagent (Invitrogen), following the manufacturer's instructions.

Cloning of the C3'H gene and its promoter

Three sets of degenerate primers (COD C3'H For, COD C3'H Nested and COD C3'H Rev, see Table 1) were designed for the amplification of C3'H from the amino acid sequences of conserved regions of *Arabidopsis thaliana* (NP_850337), *Nicotiana tabacum* (ABC69384), *Coffea canephora* (ABB83677), *Ocimum basilicum* (AAL99200), *Medicago truncatula* (ABC59086) and *Sesamum indicum* (AAL47545) homologues. PCR was performed from 25 ng template of genomic DNA following the CODEHOP strategy (Morant et al. 2002) which employs a 3 min denaturation at 94°C, followed by 20 touch-down cycles [(94°C/1 min, 70°C/2 min (reducing by 1°C each cycle) and 72°C/ 2 min], and 29 cycles of 94°C/ 1 min, primer annealing temperature/ 1 min and 72°C/10 min, and finishing with a 10 min incubation at 72°C. The PCR fragment, A-tailed by the use of Taq DNA polymerase (Promega), was extracted from the agarose gel and cloned into the p-GEMTeasy vector (Promega, Madison, WI). Plasmids from two colonies were sequenced using ABI310 capillary sequencer (Applied Biosystem). To assign a putative function, a BLAST search was performed against Viridiplantae GenBank database. To obtain a complete cDNA sequence, the SMART RACE cDNA amplification kit (Clontech) was employed, following the manufacturer's instructions, except for the use of an annealing temperature 5-10°C less than was recommended. Specific primers were designed for 3' and 5' amplification of the C3'H transcript, based on the partial cDNA sequence. Fragments of the expected size were extracted from an agarose gel, cloned into the p-GEMTeasy vector and sequenced. The CYP number

of the isolated gene was provided by Dr. Nelson (<http://drnelson.utm.edu/CytochromeP450.html>). Multiple global sequence alignments were performed using ClustalW (<http://www.ebi.ac.uk/clustalw/>), applying default parameters. Phylogenetic analysis was performed using MEGA v3.0 (Kumar et al. 2004). Protein sequences used for alignments were: CYP98A1 (*Sorghum bicolor*, AAC39316), CYP98A2 (*Glycine max*, AAB94587), CYP98A3 (*Arabidopsis thaliana*, NP_850337), CYP98A4 (*Oryza sativa*, AAU44038), CYP98A6 (*Lithospermum erythrorhizon*, AB017418), CYP98A8 (*Arabidopsis thaliana*, NP_177594), CYP98A9 (*Arabidopsis thaliana*, NP_177595), CYP98A10 (*Triticum aestivum*, CAE47489), CYP98A11 (*Triticum aestivum*, CAE47490), CYP98A12 (*Triticum aestivum*, CAE47491), CYP98A13v1 (*Ocimum basilicum*, AAL99200), CYP98A13v2 (*Ocimum basilicum*, AAL99201), CYP98A14 (*Solenostemon scutellarioides*, CAD20576), CYP98A19 (*Pinus taeda*, AAL47685), CYP98A20 (*Sesamum indicum*, AAL47545), CYP98A21 (*Ammi majus*, AAT06912), CYP98A27 (*Populus trichocarpa*, ACC63870), CYP98A28 (*Camptotheca acuminata*, AAS57921), CYP98A29 (*Zea mays*, assembled from GSS sequences), CYP98A33v1 (*Nicotiana tabacum*, ABC69384), CYP98A35 (*Coffea canephora*, ABB83676), CYP98A36 (*Coffea canephora*, ABB83677), CYP98A37 (*Medicago truncatula*, ABC59086), CYP98A39v1 (*Triticum aestivum*, AJ585988), CYP98A39v2 (*Triticum aestivum*, AJ585990), CYP98A39v3 (*Triticum aestivum*, AJ585991), CYP98A40 (*Triticum aestivum*, AJ585989), CYP98A41 (*Triticum aestivum*, AJ585987), CYP98A46 (*Coptis japonica*, BAF98473). The Genome Walker Universal kit (Clontech) was used to isolate the globe artichoke C3'H promoter, following the manufacturer's protocol. Fragments were isolated after agarose gel separation, cloned into the p-GEMTeasy vector and sequenced. The promoter's regulatory motifs were identified [according](#) to Narusaka et al. (2004).

Heterologous expression of the C3'H gene in brewers' yeast

The isolated globe artichoke C3'H gene was amplified from cDNA template with primers Expr C3'H For and Expr C3'H Rev (Table 1), modified to introduce *Bgl*II and *Eco*RI cloning sites at the 5' and 3' ends. The amplicons were cloned into p-GEMTeasy vector and sequenced using ABI310 capillary sequencer. After

*Bgl*II and *Eco*RI digestion, the fragments were directionally cloned into the expression cassette of pYeDP60 (Pompon et al. 1996) digested with *Bam*HI and *Eco*RI. *Saccharomyces cerevisiae* strain WAT11 is a derivative from strain W303-1B, in which yeast *CPR* gene (coding for a NADPH-cytochrome P450 reductase) was replaced by the *Arabidopsis ATR1* (Urban et al 1997). WAT11 was transformed using the lithium acetate / single stranded carrier DNA / polyethylene glycol method (Gietz and Woods 2002). Transformants were selected on SGI plates (1 g/l bactocasaminoacids, 20 mg/l tryptophan, 6.7 g/l Yeast Nitrogen Base, 20 g/l glucose, 20 g/l bactoagar) and successful transformation was confirmed by PCR analysis.

Enzymatic assay of the C3'H activities

Yeast microsomes were isolated after 24 h of induction on 20 g/l galactose at 30°C, according to Pompon et al. (1996). The amount of total protein in microsomes was determined according to Bradford protein assay. The substrate specificity of microsomal fractions, extracted from the recombinant yeast culture, was tested with *p*-coumaric acid, *p*-coumaroylshikimate and *p*-coumaroylquininate (kindly provided by Dr. Ullmann, Université Louis Pasteur, Strasbourg). The negative control was represented by the microsomal fraction of yeast transformed with an empty plasmid, and the positive control from yeast expressing CYP98A3 (orthologous of C3'H in *A. thaliana*, obtained from Arabidopsis Biological Resource Center Arabidopsis, Ohio State University). C3'H enzymatic activity was measured in 100 µl reaction tubes containing 600 µM NADPH, a range of *p*-coumaroylshikimate concentrations (0, 2, 5, 8, 10, 15, 20, 40, 60, 80, 100 and 150 µM), 100 mM sodium phosphate buffer (pH 7.4) and 30 µg of total microsomal proteins. The same conditions were applied with *p*-coumaroylquininate or *p*-coumaric acid as substrate. After starting the reaction upon addition of the enzyme, the tubes were shaken in the dark at 30°C for 30 min, and the reaction was stopped by adding 100 µl of trifluoroacetic acid: methanol (1:1000 v/v). The reaction products were filtered through a 0.45 µM Anotop 10 filter (Whatman) and immediately analyzed by HPLC with photodiode array (PDA) detection, as described below.

HPLC analyses

Enzymatic assays were analyzed by reverse-phase HPLC with photodiode array detection, as described recently (Moglia et al. 2008). In short, the HPLC system comprised a Waters 600 gradient controller, a Waters 996 photodiode array (PDA) detector and a column incubator held at 40°C. For the chromatographic separation an analytical column Luna C18 (2) (2x 150 mm, 100 Å, particle size 3 µM) with a pre-column (2x 4 mm) from Phenomenex was used. The mobile phase consisted of degassed trifluoroacetic acid: ultrapure water (1:1000 v/v, eluate A), and trifluoroacetic acid : acetonitrile (1:1000 v/v, eluate B), starting at 5% B, 95% A and increasing linearly to 35% B, 65% A over 45 min. The flow rate was 1 ml/min, the injection volume 10 µl, and the range of detection wavelength was 240-600 nm. Peak areas were calculated using Empower software (Waters).

UV-C treatment, and RT-qPCR assays

Three globe artichoke foliar discs were exposed to UV-C treatment (16 W germicidal lamp during 20 min) as described by Moglia et al. (2008). They were then ground separately to a fine powder in liquid nitrogen, and RNA extraction was performed from 100 mg of each powdered leaf, as described above. Primers (RT C3'H For, RT C3'H Rev Table 1) were designed on C3'H sequence using the Primer 3 software (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi). As a housekeeping gene, actin was chosen for its stability and level of expression, which is comparable to the genes of interest and whose expression remained stable after the UV-C stress. The primers (RT Act For, RT Act Rev, Table 1) were designed on the globe artichoke actin (ACT, AM744951).

For the real time quantitative PCR (RT-qPCR), cDNA was first prepared in a 20 µl RT reaction containing 5x iScript Reaction Mix (Bio-Rad), 1 µl iScript Reverse Transcriptase and 1 µg total RNA. PCR primers were designed to detect globe artichoke C3'H and actin (Table 1). The cDNA was diluted to obtain a threshold cycle (CT) value between 25 and 35. The 20 µl RT-qPCRs, performed in triplicate for each sample, contained 1x iQ Supermix, 1x SYBR-Green I (iQTM, SYBR® GreenSupermix), 10 µM primer and 3 µl diluted cDNA. PCR

reactions were carried out in 48-well optical plates using the iCycler Real-time PCR Detection System (Bio-Rad Laboratories, USA). The PCR conditions comprised an initial incubation of 95°C/5 min, followed by 35 cycles of 95°C/15 s and 60°C/60 s. In all experiments, appropriate negative controls containing no template were subjected to the same procedure to detect or exclude any possible contamination. Melting curve analysis was performed at the end of amplification.

Standard curves were analyzed using iCycler iQ software. Amplicons were analysed by the comparative threshold cycle method, in which $\Delta\Delta C_t$ is calculated as $\Delta C_{tI} - \Delta C_{tM}$, where ΔC_{tI} is the C_t value for the any target gene normalized to the endogenous housekeeping gene and ΔC_{tM} is the C_t value for the calibrator, which is also normalized to housekeeping gene.

SNP detection and linkage analysis

Sequence variation in the C3'H gene was sought by comparing the copy present in the non spiny globe artichoke variety 'Romanesco C3' with the spiny type 'Spinoso di Palermo'. Parental genomic DNAs were amplified with Expr C3'H For and Exp C3'H Rev (Table 1), and the amplicons were directly sequenced to facilitate SNP mining.

SNPs genotyping was carried out with the tetra-primers ARMS-PCR method (Ye et al. 2001) by using two sets of outer and inner primers (Table 1), designed using the software made available on-line (http://cedar.genetics.soton.ac.uk/public_html/primer1.html). PCR products were separated by 2% agarose gel electrophoresis. The segregation data obtained in an established mapping population (Lanteri et al. 2006) were combined with those associated with the markers used to construct the map and used to locate the map position of C3'H gene.

Segregation data of C3'H-SNP marker were monitored and analyzed in the 94 individuals of F1 progeny together with those of 35 AFLP, 41 S-SAP, 38 M-AFLP and 51 SSR markers previously applied for globe

artichoke map construction (Lanteri et al. 2006). The goodness of fit between observed and expected segregation data was assessed using the chi-square (χ^2) test. Based on the two way-pseudo test cross mapping strategy, independent linkage maps were constructed for each parent using the double pseudo-testcross mapping strategy (Weeden 1994) by using JoinMap 2.0 software (Stam and Van Ooijen 1995). For both maps, linkage groups were accepted at a LOD threshold of 4.0. To determine marker order within a linkage group, the following JoinMap parameter settings were used: Rec = 0.40, LOD =1.0, Jump = 5. Map distances were converted to centiMorgans using the Kosambi mapping function (Kosambi 1944). Linkage groups were drawn using MapChart 2.1 software (Voorrips 2002).

Results

Gene and promoter isolation

To isolate globe artichoke C3'H coding sequences, degenerated primers were used to amplify an 800 bp fragment from leaf genomic DNA. Only one amplified fragment was obtained and showed (Blastx) high amino acid similarity (>90%) with members of the CYP98A family. The original 800 bp genomic sequence included one 171 bp intron.

After RACE-PCR, the sequence was extended to 1524bp, representing a 508 residue protein (CYP98A49, accession number: FJ225121). The globe artichoke CYP98A49 gene contains four expected P450 conserved domains: the proline rich membrane hinge (PPGP), the I-helix involved in oxygen binding and activation (A/G-G-X-E/D-T-T/S), the clade signature (PERF) and the cysteine-containing region (PFGXGRRXCX) (Fig. 2).

A phylogenetic tree was constructed using 30 sequences from CYP98 family (Fig. 3). Most of the accessions are included in two main clusters with high bootstrap probability: one comprises genes derived from Monocots species and the other one derived from Dicots and a Gymnosperm (*Pinus Taeda*) species. This division into two main groups (as previously observed by Morant et al 2007) indicates that the encoding genes resulted from early duplication of ancestral gene. As opposite CYP98A8 and CYP98A9, two other CYP98A members from *A. thaliana*, and CYP98A20 are well separated from the main clusters. Globe artichoke CYP98A49 grouped in a sub-cluster of 5 proteins, and seemed closer to CYP98A46 from *Coptis*, CYP98A27 from *Populus*, CYP98A37 from *Medicago* and CYP98A2 from *Glycine*.

In addition to the coding region, we searched the 2 kb sequence upstream of the ORF (FJ225121). The CYP98A49 putative transcription start site was 24 bp upstream of the ATG start codon. Upstream of this transcription start site are a putative TATA box (-32 bp) and a putative CAAT box (-145 bp). The promoter contains the following regulatory elements: recognition sites of MYB, MYC, DRE-core, W-Box, TGA Box, P-Box, BoxIV and WRE1 (wound responsive element 1). All these elements, except WRE1, were also detected in the promoter region of *Arabidopsis* CYP98A3 (Fig. 4).

In vitro enzymatic activity

In order to test its enzymatic activity, the CYP98A49 protein from globe artichoke was expressed in yeast. The microsomal fraction was subsequently isolated and tested for enzymatic activity *in vitro* using several substrates at different concentrations. In the presence of *p*-coumaroylshikimate (Fig. 5A), the recombinant protein synthesized a compound that could be identified as caffeoylshikimate. The enzymatic reaction was completely dependent on the presence of NADPH. The same product was generated by microsomes from yeast expressing *A. thaliana* CYP98A3 (Fig. 5B, positive control), but not by yeast expressing the empty vector (Fig. 5C, negative control).

Moreover, the enzyme was able to convert *p*-coumaroylquininate into 5-O-caffeoylquininate (=chlorogenic acid) although the reaction was much less efficient and required a highest concentration of substrate (100 μ M) to generate a detectable product. However, the low enzymatic activity hampered any accurate evaluation of enzymatic affinity. In the presence of *p*-coumaric acid, no enzymatic conversion was detected at all (data not shown).

UV induced response

The relative expression of the CYP98A49 gene in response to UV-C exposure of globe artichoke leaves was measured by RT-qPCR, using actin to normalize the expression levels. Standard curves for each amplification system (data not shown) revealed a correlation coefficient $r > 0.99$ and efficiency higher than 90%, as indicated by slope values. The level of CYP98A49 transcripts in UV-exposed leaves was 4.2 ± 0.95 (n=3) fold higher than that of the non-irradiated control leaves, indicating increased expression of this gene upon UV-C treatment.

SNP detection and linkage analysis

A comparison of the CYP98A49 genomic sequence of 'Romanesco C3' and 'Spinoso di Palermo' resulted in the identification of one nucleotide difference at position 447 (data not shown). The tetra primers ARMS-PCR assay demonstrates that both parents are heterozygous at this base position. The C3'Hsnp447 locus segregated in a 1:2:1 ratio ($\chi^2 = 2.80$, $p > 0.1$) in the mapping population (Fig. 6), and mapped to linkage group 10 in both maps, ~2 cM from the microsatellite locus CELMS-39 and 8 cM from the AFLP locus p45/m47-07 (Fig. 7). Twenty one markers were assigned to the female LG 10: four microsatellites (CELMS-04, -20, -39 and CLIB-04), two S-SAP (cyre5 markers), 2 M-AFLP (polyGA markers) and 12 AFLP together with SNP-C3'H, covering 84.4 cM and a mean inter-marker distance of 4.22 cM). The majority of map interval (70%) was less than 5 cM and three gaps of 8 cM were present. The male LG 10 was composed of 18 markers: two microsatellite (CELMS-04 and -39) one S-SAP, 14 AFLP and the SNP-C3'H, spanned 99.4 cM with a marker density of 5.84 cM. Three large gaps longer than 12 cM were detected. Nine intercross markers (comprising C3'H gene) were shared between the parents, allowing the alignment of the maternal and paternal LG 10 (Fig. 7). The CYP98A49 gene is located, in both parental and maternal maps, with a map distance of about 2 cM from the previously mapped microsatellite CELMS-39, while a 8 cM gap was detected from the AFLP marker p45/m47-07. Estimation of markers order and distance of LG10 were improved with the integration of the co-dominant SNP-C3'H markers, increasing the number of bridge markers. The relative orders of some markers previously determined (Lanteri et al. 2006) are slightly changed, as two inversions and a small shift of a few cM were detected (Fig. 7).

Discussion

The P450 proteins form a large family of enzymes involved in plant metabolism, but the function of about 80% of them remains unknown. The *A. thaliana* genome includes 273 cytochrome P450 genes distributed in 45 families and sub-families (<http://drnelson.utmem.edu/Arablincs.html>).

Globe artichoke CYP98A49 sequence is highly homologous to some of the other CYP98 genes (up to 86% of identity and 93% of similarity to CYP98A46 from *Coptis japonica*), and contains the conserved domains associated with P450 family members (Fig. 2). The 3'-hydroxylation step is critical in the synthesis of phenolic compounds. Most of the members of the CYP98 family, described to date, metabolize shikimate esters of *p*-coumaric acid more efficiently than quinate esters (Schoch et al. 2001; Schoch et al. 2006; Morant et al. 2007). On the other hand CYP98A35 from coffee is capable of metabolizing *p*-coumaroylquininate and *p*-coumaroylshikimate with the same efficiency (Mahesh et al. 2007). CYP98A49 from globe artichoke appears to show a lower affinity for quinate esters than shikimate esters (Fig. 5), but the limited activity detected for shikimate esters hampered an accurate evaluation of enzymatic activity with quinate esters.

Shikimate esters are transient intermediates in the formation of more oxygenated compounds such as lignin precursors and chlorogenic acid. Two distinct pathways have been proposed for the synthesis of chlorogenic acid: (1) pathway from *p*-coumaroyl-CoA, involving first a trans-esterification of this compound and quinic acid via hydroxycinnamoyl-CoA:quininate hydroxycinnamoyl transferase (HQT) activity and then hydroxylation of *p*-coumaroylquininate to 5-caffeoylquinic acid, catalysed by C3'H; (2) pathway involving *p*-coumaroyl-CoA trans-esterification with shikimic acid by means of hydroxycinnamoyl-CoA:shikimate hydroxycinnamoyl transferase (HCT), then *p*-coumaroylshikimate hydroxylation to caffeoylshikimate. This compound is then converted by HCT (Hoffmann et al. 2003; Comino et al. 2007) to caffeoyl-CoA which is conjugated to quinic acid by HQT (Niggeweg et al. 2004) activity, to give 5-caffeoylquinic acid.

It has been long debated whether the HQT enzyme either directly acts on caffeoyl-CoA and quinic acid to produce chlorogenic acid, or whether it synthesizes *p*-coumaroylquininate from *p*-coumaroyl-CoA and quinic

acid, which is converted to chlorogenic acid by C3'H. Strong support for the first alternative has been provided in tomato, in which silencing of the HQT gene resulted in a 98% reduction in the level of chlorogenic acid (Niggeweg et al. 2004).

The gene encoding HCT in globe artichoke has recently been isolated (Comino et al. 2007). It is not clear, in this stage, if there are other C3'H genes and for this reason is not yet possible to conclude what is the route involved in the synthesis of chlorogenic acid in globe artichoke.

Some CYP98 genes are expressed constitutively, while others (particularly those involved in the stress response) are inducible. *A. thaliana* CYP98A3 is constitutively expressed and is upregulated by wounding (Schoch et al. 2001). *Phaseolus vulgaris* CYP98A5 is inducible by treatment with either 3,5-dichlorosalicylic acid or 2,6-dichloroisonicotinic acid (Basson and Dubery 2007), while the activity of *Daucus carota* 5-O-(4-coumaroyl)-D-quinic acid 3'-hydroxylase could be greatly increased by irradiation with blue/UV light (Kühnl et al. 1987). In order to evaluate the changes of CYP98A49 expression levels in response to UV-C induction, we performed RT-PCR experiments. We have shown in a previous work (Moglia et al. 2008) that in globe artichoke the production of dicaffeoylquinic acids, which are powerful antioxidants, can be induced upon exposure to UV-C, which suggests a role for these compounds in the protection of young leaf tissue from reactive oxygen species generated by excess light. The expression level of CYP98A49 gene was strongly increased (>4 fold higher) in UV-C treated leaves, as compared to non-treated control leaves, thus indicating not only activation in response to UV light, but likely also a putative role of this enzyme in dicaffeoylquinic acid accumulation.

Transcription factors are important in the regulation of plant responses to environmental stresses. Most of cytochrome P450 genes induced by abiotic and biotic stresses contain the recognition sites of MYB, MYC, TGA-box and W-box for WRKY factors in their promoters (Narusaka et al. 2004). The sequence analysis of the upstream region of globe artichoke CYP98A49 gene revealed the presence of most of these regulatory regions (Fig. 4). Moreover, the same kind of motifs was found in the promoter of *Arabidopsis* CYP98A3 (not tested for UV response), which is homologous to the globe artichoke CYP98A49 gene. In a previous work

(Narusaka et al. 2004) the distribution of cis-acting elements in the regulatory region of P450 *Arabidopsis* genes activated in response to UV-C radiation, was analyzed. Interestingly, all the UV-induced P450s in *Arabidopsis* share with the globe artichoke CYP98A49 promoter the recognition sites of MYB, MYC and the binding site of WRKY factors (W-box). Therefore, it is possible that the cis-acting elements recognized by MYB, MYC and WRKY transcription factors may regulate the expression of genes induced upon UV-C.

The identification of the genetic basis of metabolite variation in *A. thaliana* has been pioneered by Keurentjes et al. (2006), by applying quantitative trait loci (QTL) analyses on a large metabolomics data set. This approach, if applied to crop species, may lead to the development of informative genetic markers that could be exploited in breeding programs aimed at increasing the level of specific phytochemicals. The *C. cardunculus* genome is yet poorly mapped. In order to move to a crossing strategy for breeding, a greater knowledge of artichoke genome will be essential. In particular it will be advantageous to establish a framework of linkage relationships for reaching a better knowledge of the genetic bases of the phenylpropanoid pathway. The linkage relationships we established for the globe artichoke CYP98A49 gene may thus represent an initial step in this direction (Fig. 7). The precision of both marker order and inter-marker distances of LG10 has been improved with the integration of CYP98A49 gene.

Future efforts of our research will go in the direction of studying the role of the CYP98A49 gene in globe artichoke development, by means of forward genetic approaches, and in the identification of QTLs associated with the production of phenolic compounds such as chlorogenic acid and dicaffeoylquinic acids. Indeed we are proceeding to the construction of genetic maps based on F1 populations involving combinations between 'Romanesco clone C3' with either cultivated as wild cardoon accessions; these populations will allow comparative QTL mapping studies.

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Tables

Table 1: Primer sequences used in this study for gene isolation (COD), for heterologous expression (Expr), for genetic mapping (C3'H447) and for RT-qPCR (RT).

COD C3'H For	5'-GATGARGTYACHGCIATGGTTGA-3'
COD C3'H For Nested	5'-GAATGGGCNATGGCVGA-3'
COD C3'H Rev	5'-ATATCRTARCCHCCRAT-3'
Expr C3'H For	5'-ATGACCCTCCTACTCCTCCCC-3'
Expr C3'H Rev	5'-TTACACATCCACGGCCACACG-3'
C3'H 447 Outer For	5'- AGTTGTTTTCTCCAAGAGGCTTGAGGC-3'
C3'H 447 Outer Rev	5'- AGTAATGGATGGGTCACCTACCCAACCC-3'
C3'H 447 Inner For	5'- GAACGGAATGAGGATCAAACGTAGTTATCG-3'
C3'H 447 Inner Rev	5'- GAACGGAATGAGGATCAAACGTAGTTATCG-3'
RT C3'H For	5'- CTCTATCAGCGCCTCCGATT-3'
RT C3'H Rev	5'- ATATGATCGGGCCGTATTGC-3'
RT Act For	5'-TACTTTCTACAACGAGCTTC-3'
RT Act Rev	5'- ACATGATTTGAGTCATCTTC-3'

Figure legends

Fig. 1: Coumaroyl-ester metabolism affected by recombinant CYP98 genes. Conversion of a) *p*-coumaroylquininate to caffeoylquinic acid (chlorogenic acid), b) *p*-coumaroylshikimate to caffeoylshikimate.

Fig. 2: Amino acid sequences of globe artichoke CYP98A49 (FJ225121), *Arabidopsis* CYP98A3 (NP850337) and coffee CYP98A36 ([ABB83677](https://doi.org/10.1007/s11464-011-9999-9)). The P450 conserved domains are surrounded in boxes.

Fig. 3: Neighbour-joining tree phylogenetic analysis of globe artichoke CYP98A49. The length of the lines indicates the relative distance between nodes. The list of cytochrome p450s updated by Dr. Nelson at <http://drnelson.utmem.edu/CytochromeP450.html> was the starting point for the analysis.

Fig. 4: The localization of *cis*-acting elements in the promoter region of CYP98A3 of *Arabidopsis* (At) and of globe artichoke CYP98A49 (Cc). In particular are shown recognition site of MYB (Mb), MYC (Mc), DRE-core (D), W-box (W), TGA box (T), P-Box (P), BoxIV (IV), WRE-1 (Wr).

Fig. 5: HPLC-PDA analysis of reaction products of yeast microsomes containing globe artichoke CYP98A49 (A) or *A. thaliana* CYP98A3 (B) using *p*-coumaroylshikimate as a substrate (50 μ M). Control reactions were derived from yeast transformed with the empty plasmid (C). Chromatograms are recorded at 312 nm. Peak X1 is the substrate; while the other peaks close to it (X2, X3) represent other isomers (3 and 4) of *p*-coumaroylshikimate. The injection volume was 10 μ l.

Fig. 6: Single nucleotide polymorphism segregation in a mapping population, as detected by tetra-primers ARMS-PCR on agarose gel. Ro ('Romanesco C3') and Sp ('Spinoso di Palermo').

Fig. 7: Linkage group (LG) 10 of the globe artichoke varietal types 'Romanesco C3' (female parent, white LGs on the left) and 'Spinoso di Palermo' (male parent, grey LGs on the right). Intercross markers are shown in bold and are connected by a solid line. The LGs previously reported by Lanteri et al. (2006) are presented to

one side, and changed marker orders are indicated by dotted lines. Asterisks indicate markers showing significant levels of segregation distortion (*: $0.1 > P \geq 0.05$, **: $0.05 > P \geq 0.01$).

Figure 1

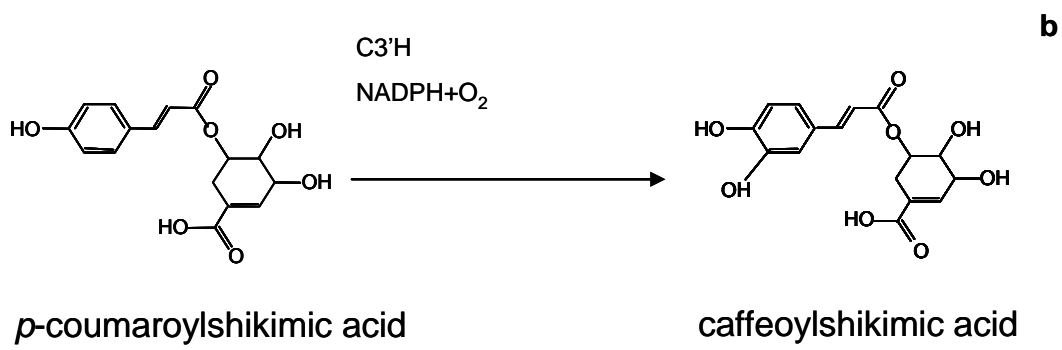
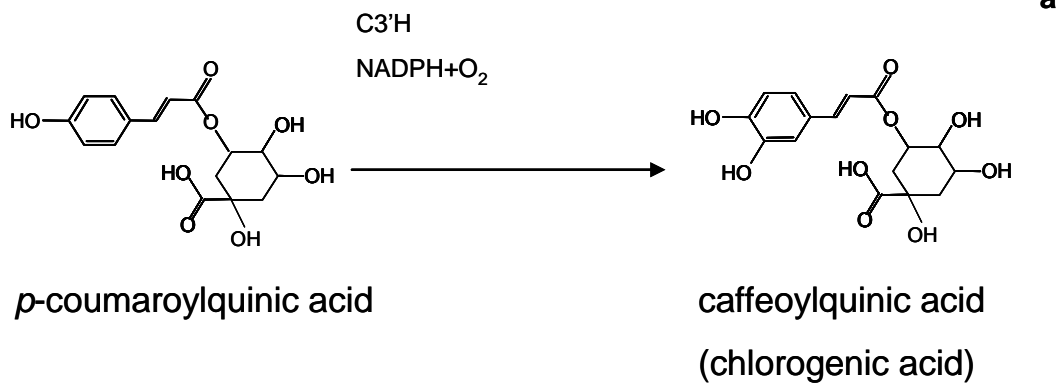


Figure 3

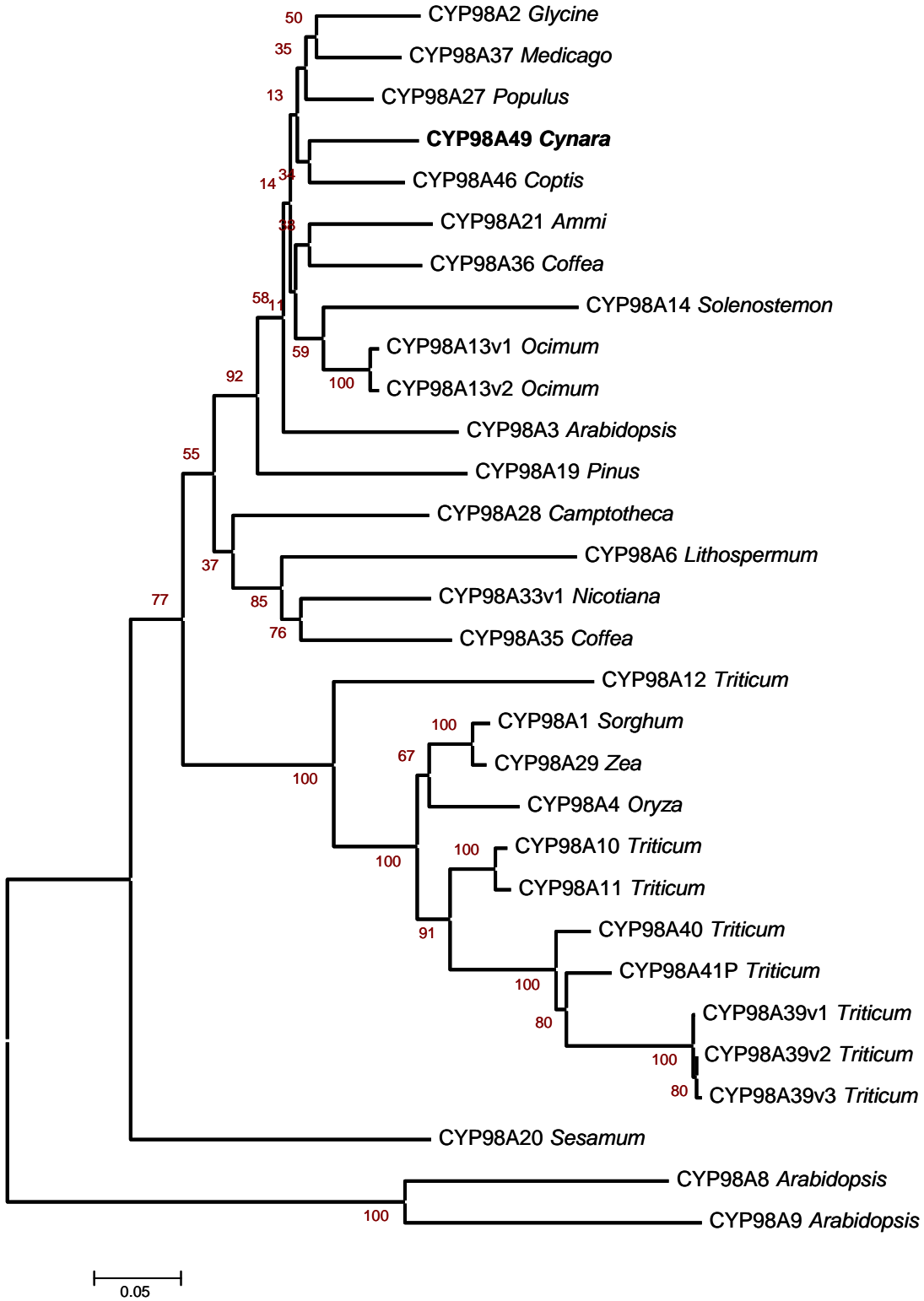


Figure 4

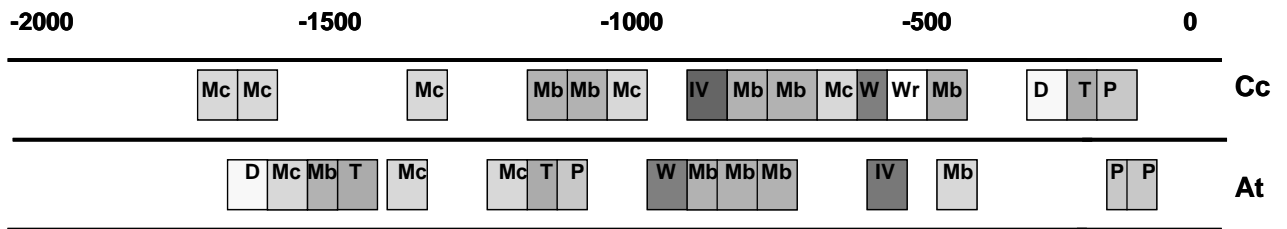


Figure 5

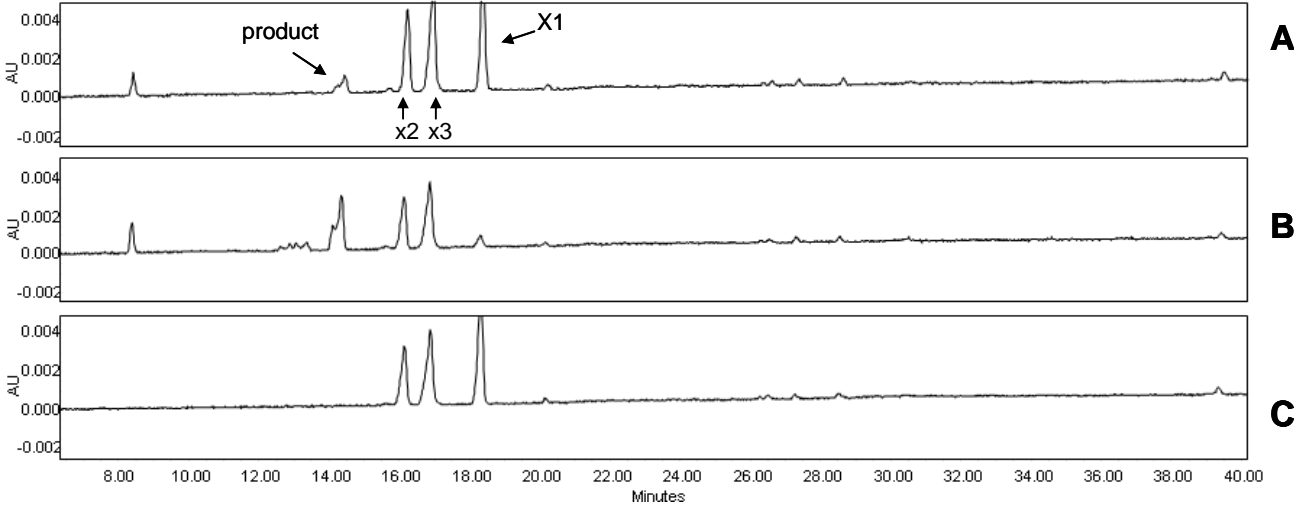


Figure 6

Sp x Ro

F1

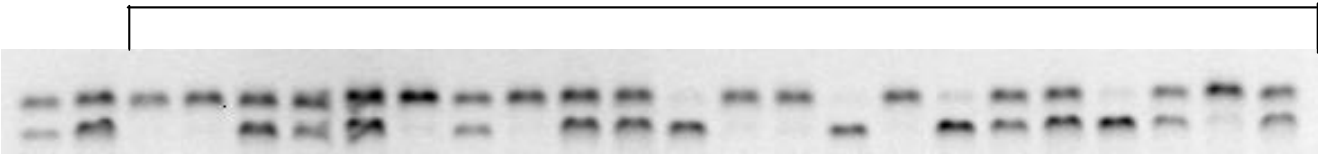


Figure 7

