

# Caffeoylquinic Acids Biosynthesis and Accumulation in *Cynara cardunculus*: State of the Art

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## Abstract

Plant secondary metabolites are highly evolved compounds performing different functions, and have been widely exploited from food to medicine. A constant supply of phenols, a class of secondary metabolites, provides preventive and defensive mechanisms to reduce the risk of chronic diseases in human beings; among them mono- and di-caffeoylquinic acids (monoCQAs, diCQAs) have attracted a growing academic and industrial interest in recent years. In *Cynara cardunculus* L. the biosynthetic pathway of chlorogenic acid (CGA, 5-*O*-caffeoylquinic acid) has been the subject of our several recent studies. Here, we report the state of the art on the isolation and in vitro functional characterization of the genes involved in the biosynthetic pathway of the CGA: HCT (hydroxycinnamoyl-CoA shikimate/quinic acid hydroxycinnamoyl-transferase), HQT (hydroxycinnamoyl-CoA quinic acid hydroxycinnamoyl-transferase), two HQT-like genes, we named Acyltransf<sub>1</sub> and Acyltransf<sub>2</sub>, and C3'H (p-coumaroyl ester 3'-hydroxylase). Plant phenolics are known to be involved in the plant stress response and we found out that in globe artichoke the exposure to UV-C induces the production of diCQAs. In UV-C treated globe artichoke leaves, the expression level of C3'H, HCT, HQT, Acyltransf<sub>1</sub>, Acyltransf<sub>2</sub> genes was strongly increased, thus confirming their involvement in the synthesis of chlorogenic acid. The development of DNA-based markers for the isolated genes made it possible to locate them within the previously developed genetic maps of the species.

## INTRODUCTION

*Cynara cardunculus* L. represents a model species for studying caffeoylquinic acids (CQAs) biosynthesis, due its exceptionally high natural content and diversity of these compounds. In various pharmacological test systems, extracts of plants with a high content of CQAs exhibit hepatoprotective, anticarcinogenic, antioxidative, antibacterial, anti-HIV, bile-expelling, as well as the ability to inhibit cholesterol biosynthesis and LDL oxidation (Rice-Evans et al., 1997; Gebhardt, 1997; Wang et al., 2003; Lattanzio et al., 2009).

The CGA biosynthesis pathway has been the target of detailed study, mainly in *Solanaceae* species (Niggeweg et al., 2004; Luo et al., 2008). Even though little direct information is as yet available concerning the biosynthesis of di- and tri-caffeoylquinic acid, the prior accumulation of CGA as precursor does appear to be necessary. Biosynthesis of CGA might occur from: (a) *p*-coumaroyl-quinic acid, synthesized by HCT (hydroxycinnamoyl-CoA: shikimate/quinic acid hydroxycinnamoyltransferase) or HQT (hydroxycinnamoyl-CoA: quinic acid HCT), and subsequently hydroxylated by *p*-coumarate-

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3'-hydroxylase (C3'H); (b) caffeoyl-CoA and quinic acid by means of HQT (Comino et al., 2007, 2009; Moglia et al., 2009; Menin et al., 2010).

The main objective of our research was to shed light on the pathway leading to the synthesis of CQAs in globe artichoke and factors affecting their content and accumulation. The acquired knowledge will make possible to identify suitable strategies for optimizing the production of CQAs in globe artichoke.

## MATERIALS AND METHODS

### Gene Isolation

Isolation of partial gene sequence of HCT, HQT and C3'H was performed by using degenerate primers and full length isolation carried out through RACE-PCR. Acyltransf\_1 and Acyltransf\_2 were identified among a set of 19,055 unigene set, using DFGWG motif as a search string to identify putative BAHD acyltransferase.

### In Vitro and In Vivo Functional Characterization

The isolated genes were first inserted in expression vectors for bacteria (pET3a-pET28) and for yeast (pYEDP60) and then expressed in heterologous host (*Escherichia coli* and *Saccaromyces cerevisiae*). Crude enzyme extracts containing recombinant proteins were tested for their enzymatic activity.

### UV-C Exposure and Gene Expression Study

Three globe artichoke foliar discs were exposed to UV-C treatment (16 W germicidal lamp, 20 min) as described elsewhere (Moglia et al., 2008). The relative expression of candidate genes in response to UV-C exposure of globe artichoke leaves was measured by RT-qPCR, using actin to normalize the expression levels.

### Gene Mapping

Sequences of target genes in parental genotypes 'Romanesco C3' and 'Spinoso di Palermo' used for map development (Portis et al., 2009), resulted in the identification of SNPs. Genotyping of SNPs was carried out through tetra primers ARMS-PCR.

## RESULTS AND DISCUSSION

### Gene Isolation and Characterization

We isolated and characterized key genes involved in the synthesis of CGA, four BAHD acyltransferases (HCT, HQT, Acyltransf\_1, Acyltransf\_2) and one P450 hydroxylase (C3'H). All the enzymes were tested for their enzymatic activity through HPLC and LC-MS analyses.

All the BAHD enzymes proved ability to use either *p*-coumaroyl-CoA or caffeoyl-CoA as an acyl donor and quinic acid as an acceptor, to generate, respectively, chlorogenic acid and *p*-coumaroyl quinate (Comino et al., 2007, 2009; Menin et al., 2010). In Figure 1 the chromatogram shows the enzymatic conversion of *p*-coumaroyl-CoA (peak 1) into *p*-coumaroyl quinate (peak 2) performed by recombinant Acyltransf\_2. In the presence of *p*-coumaroylshikimate, the recombinant C3'H protein synthesized a compound identified as caffeoylshikimate. On the contrary a lower conversion of *p*-coumaroylquinate to caffeoylquinate was detected (Moglia et al., 2009).

### Effect of Abiotic Stress on Caffeoylquinic Acid Accumulation

Secondary metabolites are involved in the plant response to environmental stresses, whether biotic or abiotic. Leaf CQA content, as for most phenylpropanoid compounds, is influenced by abiotic stress treatment, and exposure to UV-C radiation led to large increase of di-CQAs present in leaf disks of globe artichoke accessions (Moglia et al., 2008).

The gene activation in response to UV-C stress was evaluated by means of

RT-PCR. This stress affected differentially the previously isolated genes (Fig. 2). The highest increase in gene expression level was observed for HCT, that showed an enhance in transcription of 12.3-fold. The increase of gene expression upon UV-radiation not only indicates a gene induction in response to UV-stress but also an involvement of the enzymes in caffeoylquinic acid synthesis.

### Genetic Mapping

The development of DNA-based markers made possible their positioning in the *C. cardunculus* genetic maps we have previously developed (Portis et al., 2009, 2012), which were obtained by crossing a globe artichoke 'Romanesco C3' (female parent) and a cultivated cardoon 'Atilis 41' genotype (Progeny 2). At present the entire biosynthetic pathway leading to the production of chlorogenic is thus genetically mapped (Fig. 3). The integration of these gene-based markers has also improved the precision of marker order and reduced inter-marker distances in some LGs.

### CONCLUSION

We identified, characterized and mapped all the key enzymes involved in CGA biosynthesis.

Future research activities will be focused on (i) the analysis of the in vivo role of the acyltransferases by means of forward genetic approaches (e.g., gene silencing); and (ii) the identification of QTLs associated with the production of phenolic compounds.

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## Figures

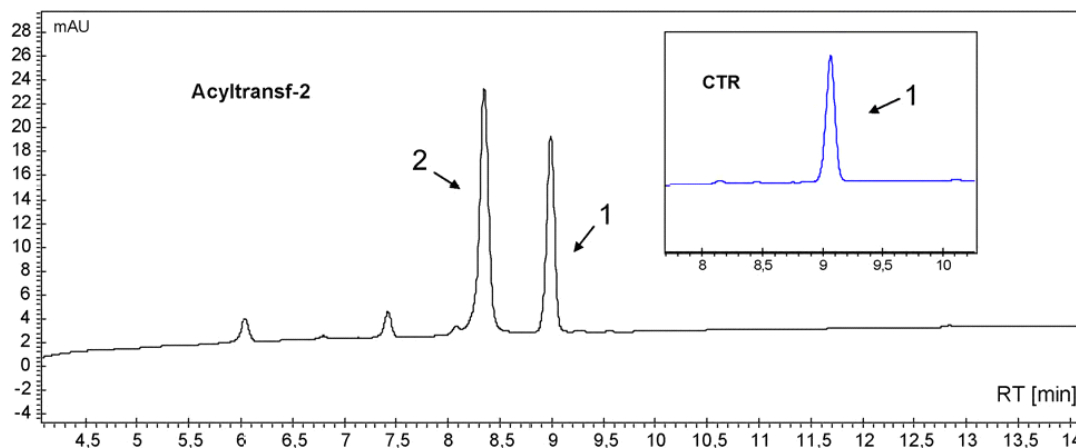


Fig. 1. Heterologous expression in *E. coli* and in vitro enzymatic assays: an aliquot of the incubation reaction without (CTR) or with (Acyltransf 2) recombinant enzyme was analysed. 1: *p*-coumaroyl-CoA; 2 *p*-coumaroylquininate.

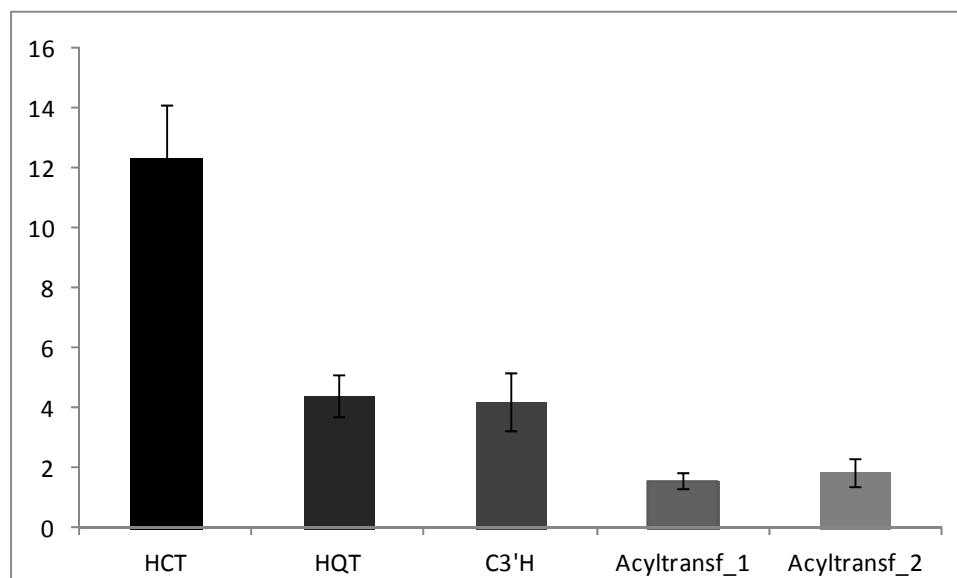


Fig. 2. RT-PCR derived patterns of expression of HCT, HQT, C3'H, Acyltransf\_1 and Acyltransf\_2 in response to UV-C radiation.

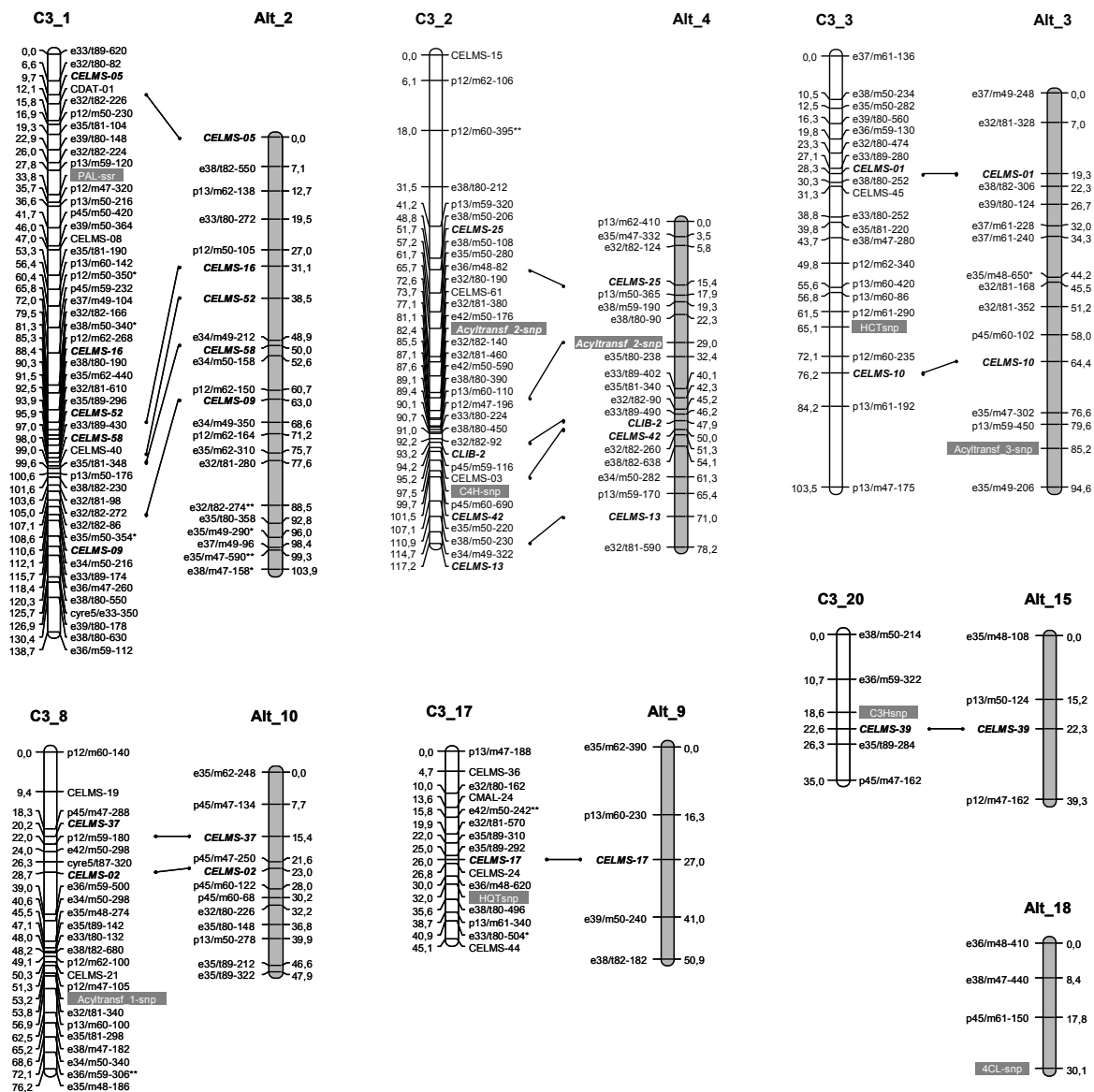


Fig. 3. Consensus linkage groups showing the location of the genes belonging to CQAs pathway. Consensus LGs of ‘Romanesco C3’ (female parent, white LGs on the left) and ‘Altilis 41’ (male parent, gray LGs on the right), incorporating the CQA biosynthesis pathway genes marked by gray boxes (taken from Menin et al., 2010).

