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Accumulation of cynaropicrin in globe artichoke and localization of enzymes involved in its biosynthesis

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ABSTRACT

Globe artichoke (*Cynara cardunculus* var. *scolymus*) belongs to the Asteraceae family, in which one of the most biologically significant class of secondary metabolites are sesquiterpene lactones (STLs). In globe artichoke the principal STL is the cynaropicrin, which contributes to approximately 80% of its characteristic bitter taste. Cynaropicrin content was assessed in globe artichoke tissues and was observed to accumulate in leaves of different developmental stages. In the receptacle, a progressive decrease was observed during inflorescence development, while the STL could not be detected in the inflorescence bracts. Almost undetectable amounts were found in the roots and inflorescence stems at the commercial stage. Cynaropicrin content was found to correlate with expression of genes encoding CcGAS, CcGAO and CcCOS, which are involved in the STL biosynthesis.

A more detailed study of leaf material revealed that cynaropicrin predominantly accumulates in the trichomes, and not in the apoplastic cavity fluids. Analysis of the promoter regions of CcGAO and CcCOS revealed the presence of L1-box motifs, which confers trichome-specific expression in Arabidopsis, suggesting that cynaropicrin is not only stored but also synthesized in trichomes. A transient expression of GFP fusion proteins was performed in *Nicotiana benthamiana* plants: the CcGAS fluorescence signal was located in the cytoplasm while the CcGAO and CcCOS localized to the endoplasmatic reticulum.

1. Introduction

Globe artichoke (*Cynara cardunculus* var. *scolymus*) belongs to the Asteraceae family. Its immature inflorescences (heads or *capitula*) are consumed as a vegetable. The inflorescences are surrounded by many bracts, of which the most inner as well as the receptacle are fleshy and edible. The leaves of globe artichoke and its related taxa, the cultivated (var. *altilis*) and the wild (var. *sylvestris*) cardoon, have been used since ancient times as herbal medicine. Therapeutic effects of *Cynara* extracts have been observed in several clinical trials [1–3] and associated with the content of phenylpropanoids (mono- and di-caffeoylquinic acids, flavonoids) and sesquiterpene lactones (STLs) [4,5]. STLs constitute a class of terpenoids which are remarkably diverse in terms of their structure and properties, which play a key role in plant environment interaction and possess allelochemical, deterrent and insecticidal properties [6,7].

STLs, which have primarily been reported from the compositae family [7,8], derive from farnesyl diphosphate and have a characteristic α-methylene-γ-lactone moiety on their C15 backbone [9]. In globe artichoke and related taxa, two predominant STLs have been described, including cynaropicrin, which contributes to approximately 80% to their peculiar bitter taste, and grosheimin, which is present in much lower amounts [10]. Cynaropicrin has been demonstrated to possess a variety of biological activities. Cho et al. [11] report an inhibitory effect on the production of proinflammatory cytokines and its immune-modulatory properties, suggesting its potential application to diseases such as virus-induced chronic inflammation and leukocyte cancer cells. In recent studies it was also evidenced that cynaropicrin prevents skin photo-aging and possesses a marked effect on mucosal injuries, preventing acute

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gastritis [12]. In addition, grosheimin was found to display a strong antioxidant and anti-allergic activity [13].

STLs, which often display cytotoxic properties, are generally separated from the cellular metabolism and stored in specialized cells and compartments such as cavities in *Solidago canadensis*, ducts in *Ambrosia trifida*, laticifers in *Lactuca sativa* or glandular trichomes in *Artemisia annua* and *Helianthus annuum* [14–17]. Glandular trichomes are specialized epidermal structures, formed by two rows of four to six cells and a terminal head. They cover the surface of many plant tissues among which *C. cardunculus* leaves [18].

The first three committed steps in the STL biosynthetic pathway have been elucidated in several *Asteraceae* species [19–23], including *C. cardunculus* [24,25], and involve three key enzymes: the terpene synthase germacrene A synthase (CcGAS in *C. cardunculus*) and two cytochrome P450s: the germacrene A oxidase (CcGAO) and the costunolide synthase (CcCOS). The objective of this work was to investigate the accumulation and localization of cynaropicrin in *C. cardunculus* and its correlation with the transcriptional expression of the three key genes involved in STL biosynthesis. In addition, the subcellular localization of CcGAS, CcGAO and CcCOS has been investigated by using confocal fluorescence microscopy.

2. Materials and methods

2.1. Plant material

Globe artichoke plants (F1 hybrid 'Concerto', Nunhems) were grown in the experimental field at Carmagnola (Torino) up to the production of commercial inflorescences (heads). Plant material was harvested at different developmental stages, as described below, and stored at −80 °C until further uses: (i) leaves from 6 weeks, 20 weeks and 1 year old plants; (ii) whole immature inflorescences at the stages before (stage 1) and after (stage 2) the development of their stem as well as at the commercial stage (stage 3), from which also the receptacle and external bracts were collected); (iii) stems of the primary head and roots at the commercial stage of the inflorescence.

Seeds of the globe artichoke Romanesco varietal types 'Concerto' and 'C3′ and of cultivated (accession 'Altilis 41′) and wild (accession 'Creta 4′) cardoon were germinated for two weeks between two layers of wetted filter paper; plantlets were then transplanted into pots in a glasshouse and grown in a climate room with 16 h day length and day/night at the temperature of 24/18 °C. Leaves from 14 weeks old plants were harvested and stored at −80 °C until further uses.

2.2. Extraction of metabolites and LC-QTOF-MS analysis

Secondary metabolite extraction and liquid chromatography-quadrupole time of flight-mass spectrometry (LC–QTOF–MS) analyses were performed according to the protocols previously described [24]. Globe artichoke plant tissues at different developmental stages (leaves, receptacle, external bracts, roots and stems), trichomes and apoplastic fluids as well as leaf tissues of globe artichoke C3 and cultivated and wild cardoon were lyophilized and ground to a fine powder in liquid nitrogen. Three biological replicates were used for each sample. Each replicate (50 mg) was extracted with 1 ml of 75% methanol and 0.1% formic acid and sonicated (125 W, 20 kHz) for 15 min. The extracts were then centrifuged at 20,000 × *g* for 5 min at 22 °C and filtered through a 0.2 μm inorganic membrane filter (RC4, Sartorius, Germany), fitted into a disposable syringe, and transferred to a glass vial. The LC-QTOF-MS platform consisted of a Waters Alliance 2795HT HPLC system equipped with a Luna C18(2) pre-column (2.0 × 4 mm) and an analytical column (2.0 × 150 mm, pore size of 100 Å, particle size of 3 μm; Phenomenex), connected to an Ultima V4.00.00 QTOF mass spectrometer (Waters, MS Technologies). Degassed eluent A, ultra-pure water: formic acid (1000:1, v/v), and eluent B, acetonitrile: formic acid (1000:1, v/v) were used at a flow rate of 0.19 mL min^{-1}. The gradient started at 5% B and increased linearly to 75% B in 45 min, afterwards the column was washed with 100% B and equilibrated at 5% A for 15 min before the next injection. The injection volume was 5 μL, the ionization was performed using an electrospray ionization source, and masses were detected in the positive mode. Absolute quantification of the cynaropicrin was performed using a standard curve of an authentic standard of cynaropicrin (Analyticon, *m/z* 347 [M^+H]). The identification of phenylpropanoids was carried out by comparing retention times and masses with those reported in our previous publication [26] based on the use of the original standards: luteolin glucoside and rutinoside from Apin (Abingdon), cynarin (1,3-dicaffeoylquinic acid) from Carl Roth (Karlsruhe) and chlorogenic acid from SIGMA. Grosheimin was identified based on its accurate mass (*m/z* 263.516 [M^+H]).

A completely randomized design (CRD) was used for the comparison of the cynaropicrin concentration, metabolites relative concentration and relative gene expression (RGE) in different tissues. Mean comparison was conducted using Duncan's test. All the data were statistically analyzed using SPSS statistical software.

2.3. Apoplast and trichome analyses

The metabolic profiles of the leaf apoplast and glandular trichomes were examined by isolating the apoplastic fluids from globe artichoke leaf tissues as described by Joosten [27]. Fresh globe artichoke leaves, sampled from the 3 weeks old plants, were submerged into water and subjected to vacuum pressure at a rate of 20 kPa. When the air bubbling from leaves started to decrease, the vacuum was released to allow the water entering into the leaves. The latter were then placed into a 10 ml plastic vessel and centrifuged immediately at 3000 g for 10 min at 5 °C. The collected apoplast fractions, the residual leaf tissues as well as not treated leaves (control) were stored at −80 °C until further analysis.

The glandular trichomes were extracted by the chloroform dipping method reported by Duke et al. [28]. Fresh young leaves from 3 weeks old plants were collected and immersed into 5 ml of chloroform for 30 seconds. The chloroform extracts were then evaporated with a stream of N_2. Chloroform dipped and non-dipped leaves (control), as well as the glandular trichome extracts, were stored at −80 °C until further analysis. Metabolic analysis of trichome was performed as described in the previous paragraph.

The different samples were examined using a Leica TCS SP2 confocal microscope equipped with 20X dry objective. Cell autofluorescence was excited at 488 nm and imaged with emission window at 500–560 nm and 560–700 nm respectively. All images were captured at 1024 × 1024 pixels.

2.4. Subcellular localization studies of CcGAS, CcGAO and CcCOS

The CcGAS, CcGAO and CcCOS genes were amplified from cDNA using the primer sequences (listed in Supplemental Table S1) and recombined into the pDONR207 Entry vector through a Gateway strategy. The amplicons were cloned into pK7WGF2 [29] producing pK7-35S:GFP:CcGAS, pK7-35S:GFP:CcGAO and pK7-35S:GFP:CcCOS respectively. As a control, the vector for expression of GFP alone pK7WGF2 (under the control of the 35S promoter), and an endoplasmic reticulum-targeted GFP-KDEL construct were also agro-infiltrated in *Nicotiana benthamiana* leaves. The expression constructs pK7-35S:GFP:CcGAS, 35S:GFP:CcGAO