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BRIEF COMMUNICATION

The differential expression of the two key genes involved in fructan biosynthetic pathway in artichoke *vs.* wild cardoon improves inulin-type fructans

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Abstract

The artichoke (*Cynara cardunculus* subsp. *scolymus*) is an intriguing source of indigestible sugar polymers such as inulin-type fructans. Artichoke represents an important component of a traditional Mediterranean diet and its edible parts are a good source of many high added value compounds such as inulin, a polymer showing relevant prebiotic properties. Compared to the cultivated varieties, the wild cardoon (*C. cardunculus* var. *sylvestris*) growing naturally under harsh conditions and well-adapted to many marginal areas, could have a good potential for use in sustainable production in stressed lands. Here, we evaluated by enzymatic assay, the amount of inulin-type fructans both in artichoke and wild cardoon in the two different organs, heads and rhizomes. The expression pattern of the genes encoding the two key enzymes sucrose:sucrose 1-fructosyltransferase and fructan 1-fructosyltransferase, involved in fructan biosynthesis, have been also evaluated. Our results showed that the amount of inulin-type fructans was higher in the wild cardoon than in the artichoke heads, together with a higher expression of the two key genes involved in the fructan biosynthetic pathway. A conspicuous content of inulin-type fructans was found also in the rhizome, supporting the significant role of these compounds in the storage and in protection from cold and/or winter stresses.

Keywords: artichoke, *Cynara cardunculus*, fructan 1-fructosyltransferase, fructans biosynthesis, gene expression, inulin, sucrose:sucrose 1-fructosyltransferase.

Cynara cardunculus L. is a herbaceous perennial Mediterranean plant including the wild cardoon taxon *C. cardunculus* var. *sylvestris* L. Fiori, recognized as the ancestor of both taxa *Cynara cardunculus* L. subsp. *scolymus* Hegi (artichoke) and *C. cardunculus* var. *altilis* DC. (cultivated cardoon) (Sonnante *et al.* 2007, Gatto *et al.* 2013).

In Mediterranean regions, artichoke represents an important component of a traditional Mediterranean diet (Rondanelli *et al.* 2013) and its edible parts (heads or capitula) are a good source of a many valuable compounds such as pectin, phenolic acid, flavonoids, and inulin (Dias *et al.* 2018, Gostin and Waisundara 2019, Zeaiter *et al.* 2019, Shallan *et al.* 2020). Also, the wild cardoon, growing naturally under harsh conditions and well-adapted to many marginal areas, could have a good potential utilisation for sustainable production in stressed lands and in maintaining the agrobiodiversity. The wild species are extremely rich resources of useful genes not available in the cultivated gene pool. Moreover, the wild cardoon is extremely rich in highly bioavailable polyphenols and high-quality fibres and minerals (Ceccarelli *et al.* 2010,

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Abbreviations: DP - degree of polymerization; FAZYs - Fructan Active enZYmes; 1-FFT - fructan 1-fructosyltransferase; 1-SST - sucrose:sucrose 1-fructosyltransferase; WSC - water soluble sugar.
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Gostin and Waisundara 2019, Muto *et al.* 2021) but, despite its interesting nutraceutical properties, to date the wild cardoon shows only a few and local applications for human foods (Christaki *et al.* 2012). In the Mediterranean areas both the taxa have an autumn-spring growing season, from September (emergence) to July (seed maturity); like the other members of the *Asteraceae* family, both artichoke and wild cardoon synthesize and accumulate fructans and also inulin, a linear polysaccharide consisting of β-(2-1) linked fructofuranosyl units with a terminal glucose unit (Raccuia and Melilli 2004, 2010). The role of fructans/inulin in plants is mainly as a reserve carbohydrates as well as membrane stabilizers and mediators of stress tolerance and drought resistance (Livingston *et al.* 2009, Van den Ende and El-Esawe 2014). In humans, the assumption of inulin-type carbohydrates results in helpful insulin resistance and glycemic control in type 2 diabetes mellitus (Rao *et al.* 2019, Wang *et al.* 2019) and improves probiotic survival (Iraporda *et al.* 2022). Further, inulin-type fructans also affect resistance to infections, lipid homeostasis, obesity and osteoporosis, reduce blood glucose and cholesterol concentration and enhance the absorption of Ca^{2+} and Mg^{2+} (Zeaiter *et al.* 2019, Tawfick *et al.* 2022).

Inulin biosynthesis is modulated by the cooperation of a group of enzymes (FAZYs; Fructan Active enZYmes) known as fructosyl transferases; two key enzymes are sucrose:sucrose 1-fructosyltransferase (1-SST) and fructan 1-fructosyltransferase (1-FFT). 1-SST modulates the initiation of inulin biosynthesis by transferring a fructose unit from sucrose to the fructosyl residue of another sucrose molecule *via* β(2-1) linkages resulting in the production of the trisaccharide 1-kestose (G1-2F1-2F); then, 1-FFT catalyses the chain elongation by transferring fructose units from and to 1-kestose or larger fructans, allowing the synthesis of both oligofructose and inulin, with a variable degree of polymerization (DP). Furthermore, fructan 1-exohydrolases (1-FEHs) mediate the degradation of inulin by removing the terminal fructose with the formation of lower-DP inulin (Kusch *et al.* 2009). Remarkably, the concentration and the inulin profile as DP vary greatly among species (Vergauwen *et al.* 2003); however, inulin molecules with a chain length up to 200, which is the highest degree of polymerization of inulin molecules known in plants, have been found in artichoke (Lattanzio *et al.* 2009, Cavini *et al.* 2022). Recently, it was also demonstrated that long chain inulins (DP 10 - 60) exert a higher immune response in humans than oligosaccharides with DP < 25 (De Vos *et al.* 2017).

In this study, we analysed inulin-type fructans in artichoke (*C. cardunculus* var. *scolymus* cv Brindisino) *vs*. wild cardoon (*C. cardunculus* var. *sylvestris*) and we evaluated the expression pattern of the two key genes for inulin biosynthesis, *1-SST* and *1-FFT*, in heads and rhizomes, respectively, to propose a suitable inulin production system. Specifically, the final aim was to detect some variations between the two taxa and/or organs and also to highlight if the wild population could represent a suitable crop for high nutraceutical properties as well as a sustainable agri-food economic system in the Mediterranean marginal areas.

Heads and rhizomes of both artichoke and wild cardoon were collected at the end of April at Firmo (39°43'N and 16°10'E), Calabria, Southern Italy. At this latitude, the plant development corresponded to growth stage 5 (inflorescence emergence and head development) as described in Archontoulis *et al.* (2010). The materials were processed for both sugar quantification and molecular analysis as reported below. Sample pooling methodology was applied in all the analyses, as an alternative approach to biological replicates (Peng *et al.* 2003, Karp and Lilley 2009); all the analyses were performed in triplicate.

The carbohydrate extraction method for both artichoke and wild cardoon was performed following Lingyun *et al.* (2007) with minor modifications. Briefly, grinded and homogenised samples were extracted in hot water (70°C, ratio 1:10) by an ultrasonic bath (37 Hz, 100 power, 30 min). The aqueous phase was filtered through a Buchner filter (43/48 µm) and the filtrate was precipitated overnight by adding of 2 volumes of 100% (v/v) ethanol and then centrifuged (6 000 *g*, 15 min). The collected pellets were completely evaporated overnight, resuspended in water and freeze-dried to recover fructans. To evaluate the content of glucose, fructose, sucrose, and fructans, supernatant and resuspended pellets were evaporated to dryness (Cavini *et al.* 2022). As described in previous literature (Zeaiter *et al.* 2019) the content of glucose, fructose and sucrose (water soluble sugar, WSC) was determined by an enzymatic kits (*Megazyme*, Wicklow, Ireland). The assay involves the conversion of sucrose, fructose and glucose into glucose 6-phosphate. Free sugars were quantified by NADPH quantification using a hexokinase/phosphoglucose isomerase/glucose 6-phosphate dehydrogenase reaction and then measured at 340 nm (Muir *et al.* 2007). The fructans were quantified by a second enzymatic assay (*Fructan HK*, *Megazyme*). Content of inulin-type fructans was calculated considering fructose, glucose, and sucrose content, measured as described above, in the extracts before and after hydrolysis through fructanase. Total fructan content was determined after hydrolysis to D-fructose and D-glucose by endo- and exo-inulinases. Total fructan content was calculated as the difference between samples treated with and without inulinase. Each sample was analysed in triplicate. Results are reported as g of sugar g^{-1} of fresh mass (FM) expressed as the mean ± SD. The asterisk indicates significant pairwise differences using Student's *t*-test (* $P < 0.001$).

Total RNA was extracted and purified starting from 100 mg of frozen and grinded tissues, using the *RNeasy* mini kit (*Qiagen*, Hilden, Germany) according to the manufacturer's instructions. RNA integrity was evaluated by electrophoresis on 0.8% (m/v) agarose gel and the final concentration of isolated RNA was determined by *ND-1000 UV-Vis* spectrophotometer (*NanoDrop Technologies*, Wilmington, DE, USA). From each RNA sample, 1 µg was treated with DNase I (DNase I recombinant, RNase-free) (*Roche*, Basel, Switzerland) and subsequently, the cDNA synthesis was performed by *SuperScript™ III*

using oligo-dT primers (*Invitrogen*, Carlsbad, CA, USA) according to the manufacturer's protocol.

Quantitative real-time PCR was performed using a *STEP ONE* instrument (*Applied Biosystems*, Foster City, CA, USA) in a 20 mm^3 of final reaction volume containing: 1× *Select SYBR® Green PCR Master Mix* (*Applied Biosystems*), 0.2 µM of each primer, 50 ng of cDNA template, and sterile double distilled water. All reactions were run in triplicate in 48 well reaction plates and negative controls were included. The cycling parameters were: 50°C for 2 min, 95°C for 2 min followed by 40 cycles of 95°C for 15 s and 60°C for 1 min. At the end of the reaction, the existence of a unique PCR product was confirmed, evaluating the melting curve by an increase of temperature of 0.5°C every 10 s, from 60 to 95°C.

Gene specific primers for *C. cardunculus* were designed using *Primer Express™* software *v3.0.1* (*Applied Biosystems*). The artichoke *1-SST* gene (GenBank acc. no. Y09662.1) forward primer 5'-CGA-GATAGGCACAGCAACAC-3', reverse primer 5'-GCT-TCAAGCGTCGATTCCAA-3' and artichoke *1-FFT* gene (GenBank acc. no. AJ000481.2) forward primer 5'-GGAAGGTTCTTTGCGTCGAA-3', reverse primer 5'-CGTCTTGGTCGTAACTGTCG-3', were used. The artichoke *ACTIN* gene (GenBank acc. no. AM744951.1) forward primer 5'-TGCTGGATTCTGGAGATGGT-3' and reverse primer 5'-ATCAAGACGGAGGATGGCAT-3' was used as reference gene for data normalization, according to Maroufi *et al.* (2018). The average efficiency of all the used primer pairs ranged between 95 and 98%.

The results were analysed by *STEP One Software 2.0* (*Applied Biosystems*) using the $2^{-\Delta\Delta Ct}$ method (Schmittgen and Livak 2008). The results represent the mean value ± standard deviation (SD) and the asterisk indicates significant pairwise differences using Student's *t*-test (* $P<0.01$).

The relative expression of the two FAZYs, *1-SST* and *1-FFT* genes along with their corresponding metabolites (fructans, glucose, fructose, sucrose) were assessed in both artichoke and wild cardoon sampled at stage 5 (inflorescence emergence and head development) (Fig. 1 Suppl.). Inulin-type fructans were present in the rhizomes of both taxa with a slightly lower content in the wild cardoon [36.0 ± 4.4 g g^{-1}(FM)] than in the artichoke rhizome [96.0 ± 2.8 g g^{-1}(FM)]; conversely, a much higher amount was detected in the wild cardoon [89.4 ± 13.0 g g^{-1}(FM)] than in the artichoke heads [13.5 ± 2.4 g g^{-1}(FM)] (Fig. 1*A*). The content of water soluble sugars glucose, fructose, sucrose, detected by *Megazyme* kit, showed overall a significantly higher content in wild cardoon than in artichoke (Fig. 1*B-D*). The sucrose and glucose content was higher in wild cardoon than in artichoke, both in the heads as well as in the rhizome (Fig. 1*B*). In particular, a much higher sucrose content in the wild cardoon [32.1 ± 3.0 g g^{-1}(FM)] than in artichoke heads [4.9 ± 1.0 g g^{-1}(FM)] was detected (Fig. 1*D*), whereas the differences in the rhizome sucrose content were not significant (Fig. 1*D*). It could be hypothesized that in the wild cardoon, during this vegetative phase of growth, sucrose is synthesized in excess and translocated to the underground organs where it is metabolized into inulin, as evidenced by the transcriptions of *1-SST* and *1-FFT* genes which were significantly higher

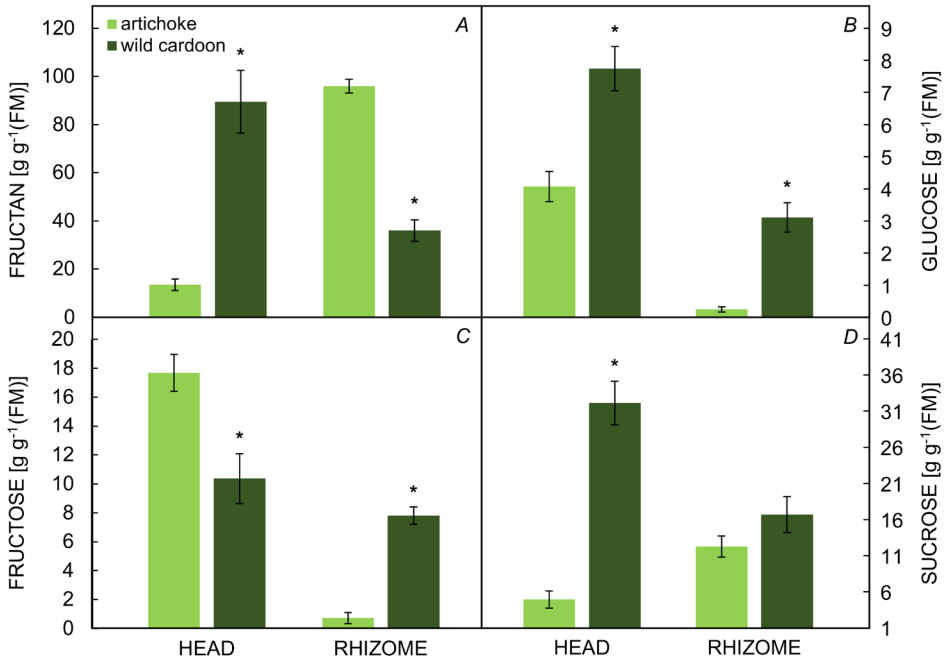

Fig. 1. Content of fructans (*A*), glucose (*B*), fructose (*C*), sucrose (*D*) in heads and rhizomes of artichoke and wild cardoon sampled at stage 5. Means ± SDs, $n = 3$, * indicates significant difference at $P < 0.001$ between the artichoke and wild cardoon.

in wild cardoon than globe artichoke head (Fig. 2). As far as concerns the fructose content, it was significantly higher in the wild cardoon [7.8 ± 0.6 g g^{-1}(FM)] than in artichoke rhizome [0.7 ± 0.3 g g^{-1}(FM)] (Fig. 1*C*); on the contrary the head artichoke showed a slightly higher fructose content [17.6 ± 1.2 g g^{-1}(FM)] than wild cardoon head [10.4 ± 1.0 g g^{-1}(FM)] (Fig. 1*C*).

The transcriptions of *1-SST* and *1-FFT* were generally higher in heads than in rhizomes and significantly higher in wild cardoon head than in globe artichoke head (Fig. 2); conversely the low expressions of both genes observed in the rhizomes were not statistically significant between the two taxa (Fig. 2).

The high activity of 1-SST and 1-FFT enzymes associated with a higher content of fructans in the wild cardoon head, supports the hypothesis that fructans are produced by the action of the 1-SST/1-FFT enzymes; whereas in the rhizome, where the expression of both *1-SST* and *1-FFT* genes was low, fructans are probably the residue of the storage material that occurred in these organs during the resting season. Indeed, in some plants, an accumulation of fructans is observed under severe drought and cold stress, since they are resistant to crystallization at low temperatures (Krasensky and Jonak 2012); additionally, acting as a source of reserve carbohydrates, they can be used during the recovery phase after stress (Konstantinova *et al.* 2002). Moreover, the high availability of WSC in the wild cardoon, depending mainly on the release of glucose and sucrose, can be explained by the increase in 1-SST enzyme activity (Cairns 1993); in fact, a good correlation between the content of free glucose (one of the products of 1-SST) and the expression of *1-SST* gene was demonstrated by Van den Ende *et al.* (1996). Because the exact regulatory pathways that control metabolism of fructans are not fully known, they are the subject of ongoing research in several laboratories. It is reasonable to expect that the pattern of storage polysaccharide accumulation is a dynamic process intimately interconnected with those controlling the storage organ differentiation (in this case the rhizome) and

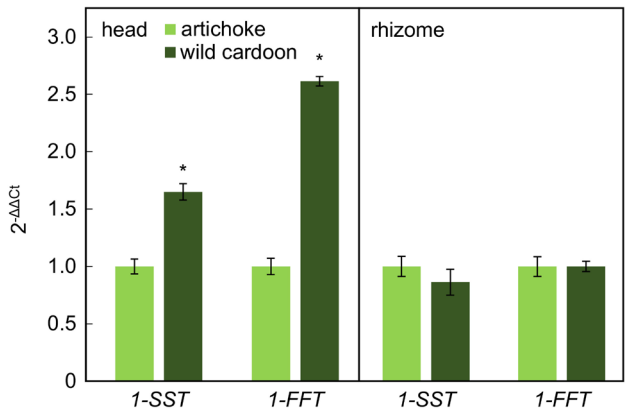

Fig. 2. Expressions of the two key FAZYs genes *1-SST* and *1-FFT* in head and rhizome of artichoke and wild cardoon sampled at stage 5. Means ± SDs, $n = 3$, * indicates significant difference at $P < 0.001$ between the artichoke and wild cardoon.

plant growth. In fact, energetic substances accumulate in the rhizomes ensure the supply of compounds when a new growth cycle starts after the summer quiescence (Raccuia and Melilli 2010, Gominho *et al.* 2018). However, it can be expected that genes for fructans/inulin biosynthetic and breakdown enzymes will be also regulated developmentally by sugars, wounding, hormones, and stress factors, such as drought or low temperature (Livingston *et al.* 2009, Joudi *et al.* 2012).

It is interesting to note that at the stage of head development, the wild cardoon had more fructans than the artichoke. We can assume that this carbohydrate reserve in the form of fructans could play an important role under stressful conditions. Probably this wild genotype retains some genetic traits conferring it a greater adaptability to marginal areas and coping with stress much more than cultivated artichoke. So, the high fructan content observed during the development of the head, confer to the wild cardoon a good potential for health promoting dietary aliments and also it can be a good alternative to the common and already highly commercialized artichoke; overall the exploitation of wild cardoon for the application in both food and industrial sectors could be more promising as a suitable crop of marginal and harsh Mediterranean areas. A further interesting note is that the expressions of *1-SST*/*1-FFT* and activities of respective key enzymes may be an early marker of fructan biosynthesis and therefore a good label of production of these interesting metabolites also at the industrial level. Nevertheless, these two FAZYs may be a valuable rapid tool to identify suitable cultivars and/or more promising genotypes in breeding or conservation programs of industrial artichoke.

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