

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

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Abstract

The artichoke (*Cynara cardunculus* L. subsp. *scolymus* Hegi) is an intriguing source of indigestible sugar polymers such as inulin-type fructans. In this study the amount and the quality of inulin-type fructans have been evaluated in the cultivated artichoke (*Cynara cardunculus* L. subsp. *scolymus* Hegi) as well as in the wild cardoon (*C. cardunculus* var. *sylvestris* L. Fiori). The expression pattern of the genes codifying for the two key enzymes sucrose:sucrose 1-fructosyltransferase (1-SST) and fructan 1-fructosyltransferase (1-FFT), involved in fructans biosynthesis, have been also evaluated both in capitula (heads) and rhizomes. The results showed that the inulin-type fructans were much higher in the heads of the wild cardoon than artichoke, one together with a higher expression of the two key genes fructans codifying enzymes. A conspicuous level 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 stress. The results indicate that wild cardoon could be a good and suitable crop for inulin production as well a sustainable agri-food system in Mediterranean marginal areas.

Introduction

Cynara cardunculus L. is an herbaceous perennial Mediterranean plant including the wild cardoon taxon *C. cardunculus* var. *sylvestris* L. Fiori, recognized as the ancestor of the both two 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 a solid component of a traditional Mediterranean diet (Rondanelli et al. 2013) and its edible parts (heads or capitula) are a good source of a lot of high added value 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 in 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 crop, as 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; Gostin and Waisundara 2019; Muto et al. 2020) but, despite its interesting nutraceutical properties, to date the wild cardoon shows only 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 carbohydrate as well membrane stabilizers, 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 helpful in 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, pre-print). Further, inulin type fructans affects also

resistance to infections, lipid homeostasis, obesity and osteoporosis, reduces blood concentration of glucose and cholesterol 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:sucrose1-fructosyltransferase (1-SST) and fructan1-fructosyltransferase (1-FFT). 1-SST modulates the initiation of inulin biosynthesis by transferring a fructose units from sucrose to the fructosyl residue of another sucrose molecule via $\beta(2 - 1)$ 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). Fructan 1-exohydrolases (1-FEHs) mediates degradation of inulin by eliminating 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. 2020). Recently, it was also demonstrated that long chain inulines (DP 10–60) exert higher immune response in humans than oligosaccharide of DP < 25 (de Vos and Vogt 2017).

In this study we analysed inulin-type fructans in wild cardoon vs cultivated accession of artichoke and we evaluated the expression pattern of the two key genes 1-SST and 1-FFT for inulin biosynthesis, in heads and rhizomes, to propose a suitable inulin production system. Specifically, the final aim was to detect some that variations between the two taxa and/or organs and also to highlight if the wild population investigated could represent a suitable crop for high nutraceutical properties as well as a sustainable agrifood economic system in the Mediterranean marginal areas.

Materials And Methods

Plant material

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

Mono-disaccharides and fructans extraction

The extraction method for both artichoke and wild cardoon carbohydrates was performed following Lingyun et al. (2007) with minor modifications. Briefly, grinded and homogenised samples were extracted in hot water (70°C, d/w 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 subjected to overnight precipitation by the addition of 2 volumes of ethanol 100% v/v and then centrifuged (6000xg, 15 minutes, RT). The collected pellets were completely evaporated overnight, resuspended in water and freeze-dried to recover fructans. To evaluate the mono-disaccharides and fructans content, supernatant and resuspended pellets were evaporated to dryness (Cavini et al., 2020).

Quantification of mono-disaccharide and fructans.

As described in previous literature (Zeaiter et al. 2019) the glucose, fructose and sucrose content was determined by an enzymatic kit (Sucrose, D-Fructose and D-Glucose assay kit, Megazyme, 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, then measured at 340 nm (Muir et al. 2007). Instead, the fructan was quantified by a second enzymatic assay (Fructan HK, Megazyme, Ireland). Inulin-type fructans concentration was calculated considering fructose, glucose and sucrose content, measured as described above, in the extracts before and after hydrolysis through fructanase. Total fructans content was determined after hydrolysis to D-fructose and D-glucose by endo- and exo-inulinases. Total fructans 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 obtained from 100 g of extract expressed as the mean $\pm$ standard deviation. The asterisk indicates significant pairwise differences using Student's t-test (* $P < 0.001$).

Total RNA extraction and cDNA synthesis

Total RNA was extracted and purified starting from 100 mg of frozen and grinded tissues, using the RNeasy Mini Kit Qiagen according to the manufacturer's instructions. RNA integrity was evaluated by electrophoresis on 0,8% agarose gel, the final concentration of isolated RNA was determined by NanoDrop (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) and subsequently the cDNA synthesis was performed by SuperScript™ III using oligo-dT primers (Invitrogen Carlsbad, CA) according to the manufacturer's protocol.

Quantitative real-time PCR (qRT-PCR)

Quantitative Real-time PCR was performed using a STEP ONE instrument (Applied Biosystems, Foster City CA) in a 20 µL of final reaction volume containing: 1X Select SYBR® Green PCR Master Mix (Applied Biosystems, Foster City CA), 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 seconds 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 0,5°C every 10 s; from 60°C to 95°C.

Gene specific primers for *C. cardunculus* were designed with Primer Express™ Software v3.0.1 (Applied Biosystems, Forester City, CA). The artichoke 1-SST gene (GenBank: Y09662.1) forward primer 5'-CGAGATAGGCACAGCAACAC-3' reverse primer 5'-GCTTCAAGCGTCGATTCCAA-3' and artichoke 1-FFT gene (GenBank: AJ000481.2) forward primer 5'-GGAAGGTTCTTTGCGTCGAA-3' reverse primer 5'-CGTCTTGGTCGTAAGTGTGCG-3', were used. The artichoke ACTIN gene (GenBank: AM744951.1) forward primer 5'-TGCTGGATTCTGGAGATGGT-3' reverse primer 5'-ATCAAGACGGAGGATGGCAT-3', was used as an endogenous reference to normalize, as reported by Maroufi et al. (2018). The results were analyzed using STEP One Software 2.0 (Applied Biosystems), using the $2^{-\Delta\Delta C_t}$ method (Schmittgen and Livak 2008). The results represent the mean value ($\pm$ standard deviation) and asterisk indicates significant pairwise differences using Student's t-test (* $P < 0.01$).

Results And Discussion

Relative expression of the two FAZYs, 1-SST, 1-FFT along with their corresponding metabolite (fructans, sucrose, glucose) were assessed in both artichoke and wild cardoon sampled at stage 5 (inflorescence emergence and development of the head) (Fig. 1). The inulin-type fructans occurred in the rhizome of both taxa with a slight lower level in the wild cardoon rhizome ($64,72 \pm 7,71$ g/100g) respect with artichoke one ($80,03 \pm 7,03$ g/100g); conversely a much higher quantity was detected in the heads of the wild cardoon ($72,53 \pm 12,03$ g/100g) compared with artichoke ones ($34,03 \pm 9,85$ g/100g) (Fig. 2A). The WSC, considered as mono (glucose, fructose) and disaccharides (sucrose) fractions, showed a significative higher content in wild cardoon heads (20.70 ± 8.03 g/100g) compared with artichoke ones (14.49 ± 3.83 g/100g) (Fig. 2B); also, in the wild cardoon rhizome a higher WSC level (25.64 ± 6.05 g/100g) vs artichoke (12.95 ± 4.67 g/100g) was detected (Fig. 2B).

The transcript levels of 1-SST and 1-FFT showed generally a higher expression in heads than rhizomes with significant higher level in wild cardoon head respect with globe artichoke ones (Fig. 3); conversely the downregulation of the expression of both enzymes observed in the rhizomes was not statistically significant between the two taxa (Fig. 3).

The high activity of 1-SST and 1-FFT associated with a higher concentration of fructans in the wild cardoon head, supports the hypothesis that fructans are produced by the active action of 1-SST /1-FFT enzyme; whereas in the rhizome, where the expression of 1-SST /1-FFT enzyme are low and at a basal level, fructans are probably the residual of the storage material that in these organs occurred during the resting season. Indeed, in some plants accumulation of fructans is observed under severe drought and cold stresses, since they are resistant to crystallization at low temperatures (Krasensky and Jonak 2012); additionally, acting as a source of reserve carbohydrate, they can get utilized during the recovery phase after a 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 concentrations of free glucose (one of the products of 1-SST) with expression level of 1-SST gene was approved by Van Den Ende et al. (1996). Although the exact regulatory pathways that control fructans metabolism are not fully known and they

are a subject of ongoing research in several laboratories, it is reasonably expected that the pattern of storage polysaccharide accumulation is a dynamic process intimately interconnected with those controlling storage organs differentiation (in this case the rhizome) and plant growth. In fact, energetic substances accumulate in rhizome ensure the supply of compounds when 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, for example, drought or low temperature (Joudi et al. 2012; Livingston et al. 2009).

It is interesting to note that at the stage of head development, the wild heads cardoon is much richer in fructans than artichoke ones; we can assume that this carbohydrate reserve in the form of fructans could play an important role in stressful conditions. Probably this wild genotype retains some genetic traits conferring it a greater adaptability to marginal area and coping with stress much more than cultivated artichoke. So, the great fructans levels observed during the head development, 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 greatly commercialized artichoke; overall the exploitation of wild cardoon for the application in both food and industrial sectors could be more promising as suitable crop of marginal and harsh Mediterranean area. A further interesting note is that the expression pathway of 1-SST /1-FFT keys enzymes may be an early marker of fructans biosynthesis and therefore a good label of production of these interesting metabolites also at the industrial level; nevertheless, this two FAZYs may be a valuable fast tool to identify suitable cultivars and/or more promising genotypes in breeding or conservation program of industrial artichoke.

Declarations

The authors declare no conflict of interests.

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Author Contributions

All authors contributed to the study conception and design. Material preparation, data collection was performed by Leonardo Bruno and Antonella Muto; gene expression pattern and data analysis were performed by Michele Ferrari, Clara De Sio and Radiana Cozza; saccharide fraction extraction and data analysis were performed by Ilaria Bruno, Stefania Pagliani and Massimo Labra. The first draft of the manuscript was written by Michele Ferrari and Clara De Sio that should be considered joint first authors; all authors commented on previous versions of the manuscript; Radiana Cozza made the final versions of the manuscript. All authors read and approved the final manuscript.

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Figures

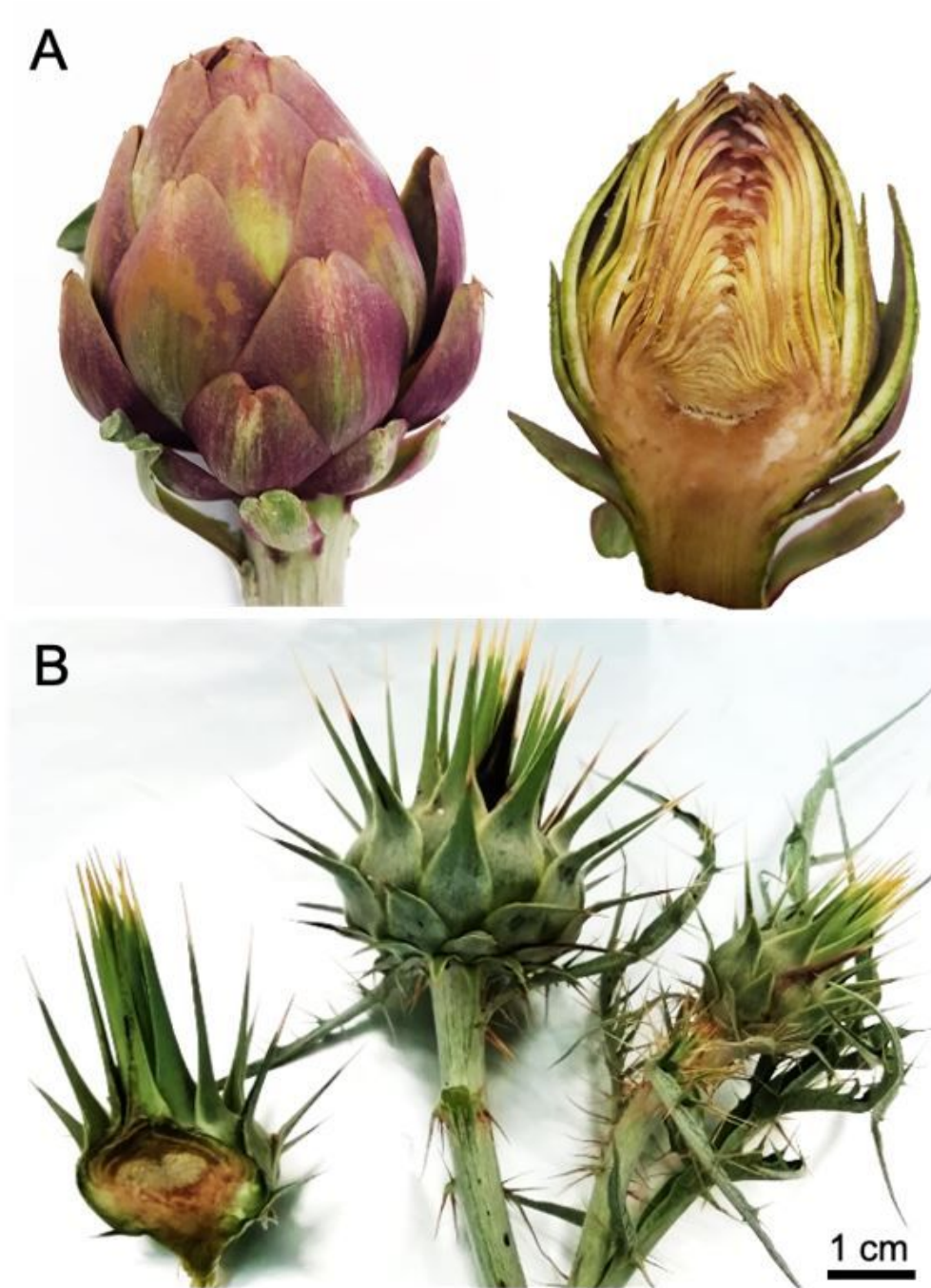


Figure 1

Phenological traits of artichoke (A) and wild cardoon (B) sampled at stage 5 (inflorescence emergence and development).

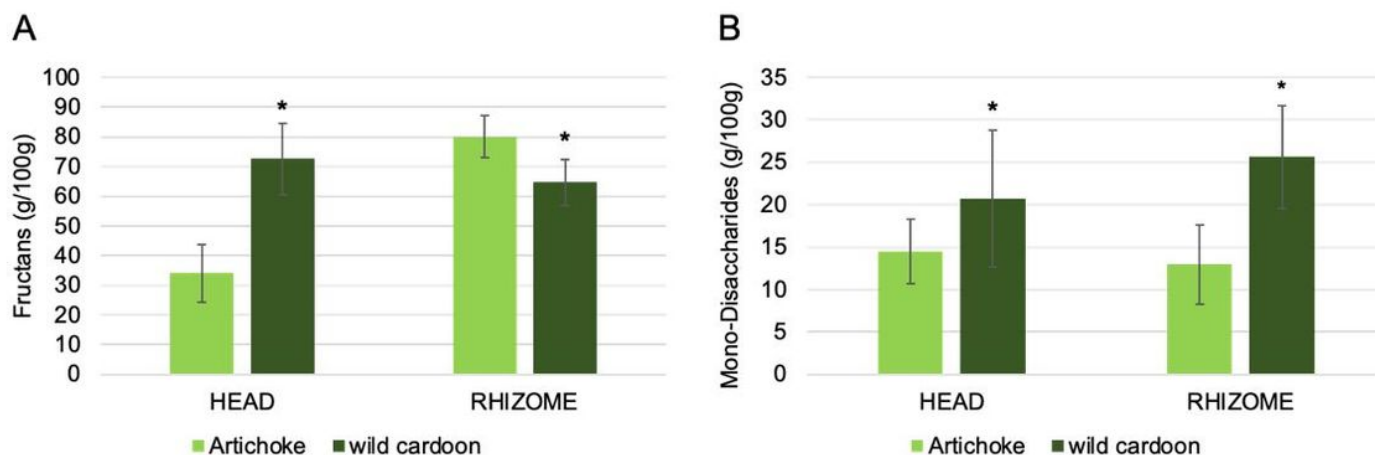


Figure 2

Fructans (A) and mono-disaccharides contents (B) (g /100g extract) in head and rhizome of artichoke and wild cardoon sampled at stage 5. Significantly different values at * $p < 0.001$.

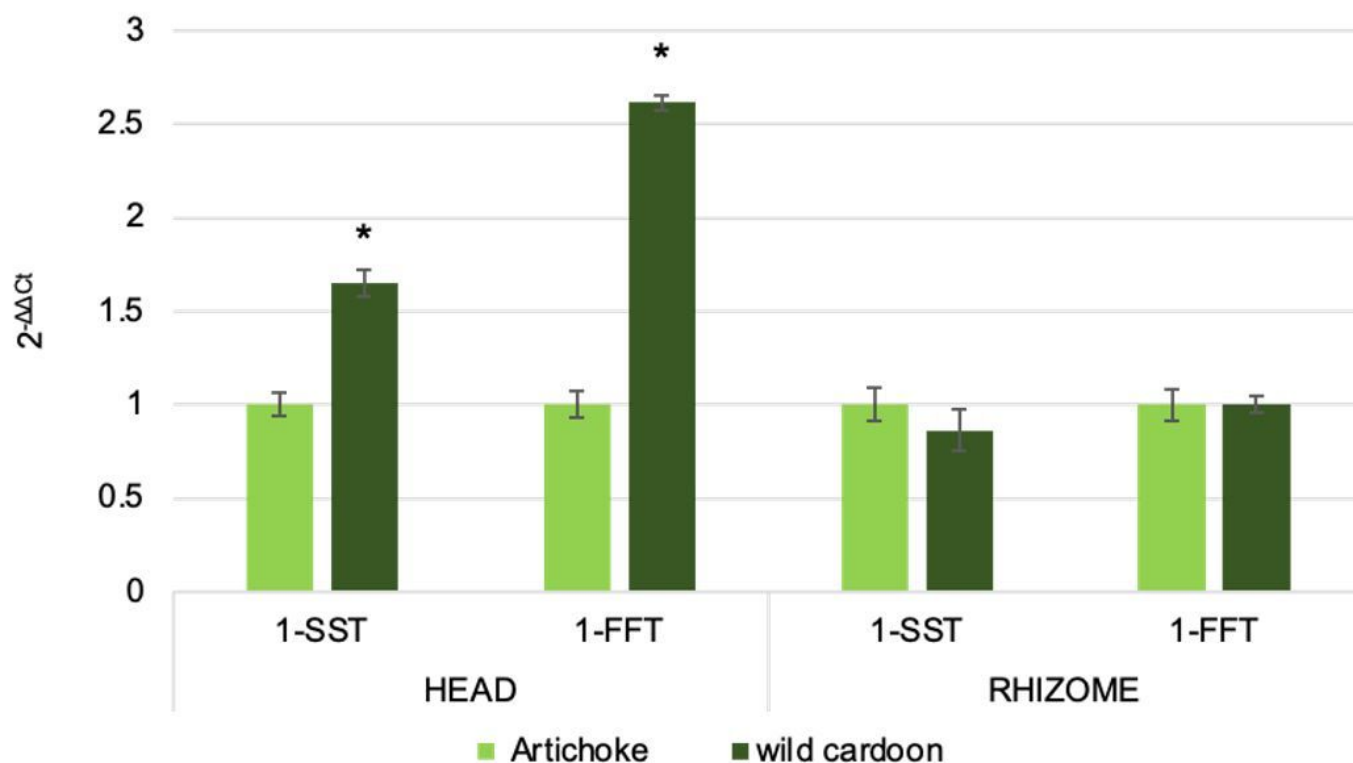


Figure 3

Expression level of the two key FAZYs genes 1-SST and 1-FFT in head and rhizome of artichoke and wild cardoon sampled at stage 5. Significantly differentially expressed level at * $p < 0.001$.