

## Spatial Expression of Genes in Inulin Biosynthesis Pathway in Wild and Root Type Chicory

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### ABSTRACT

Chicory (*Cichorium intybus* L.) is a typical Mediterranean plant distributed throughout the world and has different commercial uses such as salad, forage, inulin production, and coffee substitute. Health promoting characteristics of inulin as a prebiotic compound led to its biosynthesis pathway discovery. Two enzymes, namely, *I-SST* and *I-FFT*, are involved in inulin biosynthesis during normal phase. By cold nights or other factors, *I-FEHs* enzymes degrade inulin to fructosyl units. To compare the strength of function of these genes in a wild type genotype with root type cultivar (Orchies) of chicory at three stages, i.e. 60, 90, and 120 days after seed planting, relative expression of those genes along with their corresponding metabolites were assessed using RT-qPCR and HPLC. Expression results showed that, unlike Orchies cultivar, relative expression of *I-SST* in wild type genotype was ascending, relative expression of *I-FFT* was very low and constant and there were high levels of relative expression of *I-FEH I* gene during growing season due to flowering initiation. Also, glucose and fructose concentrations were upward, as result of *I-SST* and *I-FEH I* enzymes activity in wild type genotype, respectively. Degree of Polymerization (DP) of produced inulin had almost no increase due to low function of *I-FFT* enzyme in the wild type genotype (DP < 5), but Orchies cultivar produced inulin with DP > 10 as expression of *I-SST* decreased during growing season. So, it is possible to make inulin pathway in root type chicory cultivars more efficient by expanding and overexpressing *I-SST* function using such wild resources through backcross breeding or biotechnology methods.

**Keywords:** Industrial Chicory, Real Time PCR- Fructan, *I-SST* enzyme activity, *I-FEH I*.

### INTRODUCTION

Human beings struggle to improve the quality of life has often adversely affected his health due to limited physical activity. Diabetes type 2, constipation, obesity, and hypertension are some metabolic diseases which have necessitated addition of prebiotics, such as inulin, to the food of modern human (Roberfroid, 2005, 2007). Also, there is increasing interest in using inulin as alternative to antibiotics for poultry production (Khosravi *et al.*, 2017). While we are cognizant of the importance of inulin in creating

certain health improving effects, sugar is relegated to a much lesser role in our life. The best source for inulin production is root chicory (*Cichorium intybus* var. sativum) due its substitutability for sugar beet cultivation and industry (Haraguchi, 2011). Today, heavy weight and high inulin content of chicory root are very different from the wild types used by distilling industry, especially in Iran (Shoorideh *et al.*, 2016). Due to its usage as coffee substitute in Europe since the end of 16<sup>th</sup> century, root yield of chicory increased parallel to sugar beet until 1950s. After that, chicory root yield had no increase until 1990s (Frese *et al.*, 1991; Lucchin

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et al., 2008). Again, attempts are made to increase root yield and inulin quality through new breeding methods and inulin synthesis pathway. Inulin keeps industrial chicory going for overwintering (Pilon-Smits et al., 1995; De Roover et al., 2000) and is synthesized by action of two genes: *I-SST* that produces a trisaccharide, 1-kestose, and free glucose by irreversible interaction on two sucrose molecules, and *I-FFT* that increases Degree of Polymerization (DP) of inulin by transferring fructosyl unit from an inulin molecule as donor to another one as receiver in vacuole of parenchymal cells of root (Van den Ende et al., 1996c). Diversity among fructans and their degree of polymerization are related to this fructosyl transferase (Hellwege, 2000). Inulin fructan is divided into two categories: Common inulin (GFn) with terminal glucose and inulo-n-ose (Fn) without terminal glucose. Three kinds of fructan exo-hydrolyzing genes are determined in chicory (*I-FEH I, IIa* and *IIb*) which decrease DP of inulin by removing terminal fructose (Figure 1; Van den Ende et al, 2001). The other function of *I-FFT* appears with increasing concentration of fructosyl to produce inulo-n-ose (Figure 1). Améziane et al. (1997), without considering effects of environmental factors on inulin pathway, defined three stages of development for root chicory, i.e. young phase, mature phase, and senescence phase. As Van Den Ende et al. (1996b) mentioned, two phases for inulin pathway action can be considered (Figure 1): (i) Normal inulin pathway action phase, in which *I-SST* gene is on and produces 1-kestose and glucose and *I-FFT* produces high DP inulin. During the first phase fructose and sucrose concentrations remain constant, but high DP inulin increases due to growing *I-FFT* action, and (ii) Abnormal inulin pathway action phase, which happens by cold nights or other stresses and *I-FEH* causes inulin degradation (production of low DP inulin) and high fructose concentration (Van Den Ende and Van Laere, 1996). Although sucrose and fructose are applied to produce 1-kestose by another function of *I-FFT*, their concentrations surprisingly increases. Also, inulo-n-ose produced by *I-FFT* using fructosyl units (Van Den Ende et al., 1996b and Van Arkel et al., 2012).

Finally, quality and quantity of inulin is affected by declining of *I-SST* activity during

growing season, and the induction of *FEH* activity, which accelerates fructose production in field-grown chicory roots during autumn (Van den Ende and Van Laere, 1996; Van den Ende et al., 1996a). Relative expression based on expression ratio of these genes versus housekeeping genes, actin and elongation factor (Maroufi et al., 2010), along with investigating their corresponding metabolites would guide us to select more productive inulin pathway. Nowadays, more attention has been paid to gene expression in specific tissues at certain developmental stages and with specific phenotypes by RT-qPCR (Wang and Brown, 1999). RT-qPCR as useful tool for gene expression studies in plants (Gachon, 2004) was applied by Maroufi et al. (2012) for expression of *FEH* genes involved in inulin biodegradation in chicory cultivars during storing. There was inadequate and fragmental expression data on gene regulation related to biosynthesis and degradation of inulin in industrial chicory and almost no information about chronological regulation of inulin pathway, particularly in wild type genotypes which exist throughout the world.

Considering these information defects, the aim of the present study was to compare sequential expression ratio of inulin pathway genes in wild genotype with industrial chicory cultivar during normal phase to comprehend inulin genes functions at various stages of plant growth and detection of potential genes for any further use in breeding programs or gene manipulation.

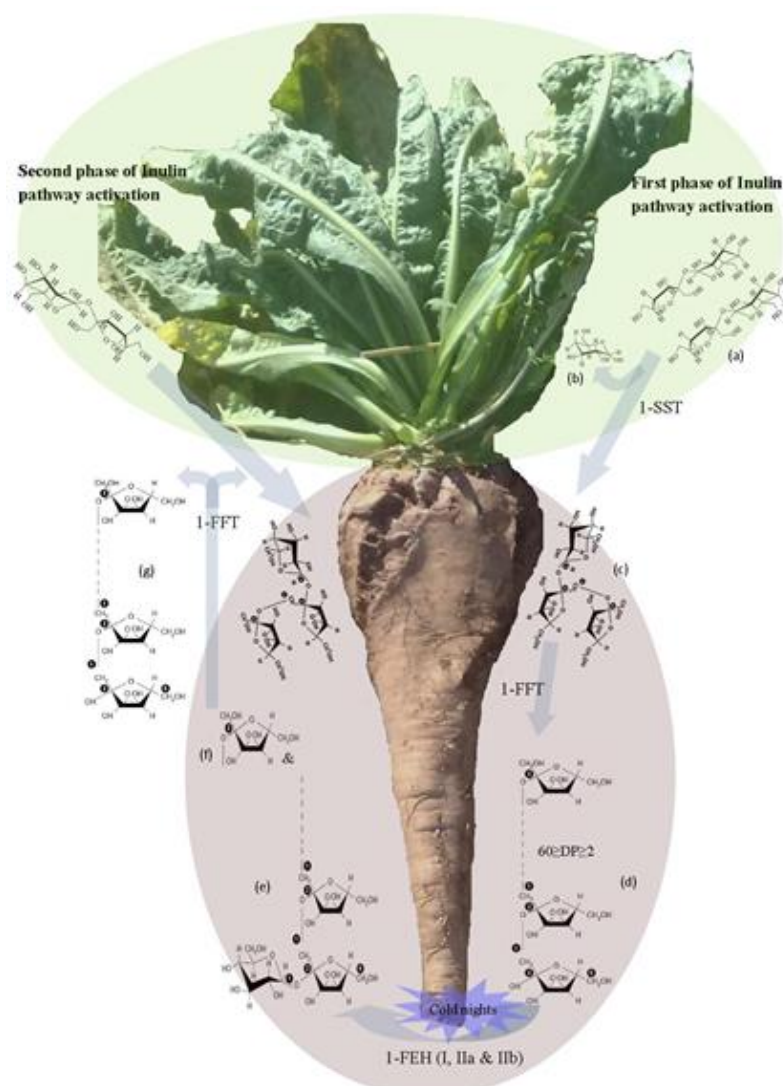
## MATERIALS AND METHODS

### Plant Materials

A chicory genotype which is cultivated for a long time in Iran for distilling (Iranian chicory genotype) was selected to compare with Orchies (UPOV, 2003) as industrial chicory variety (Table 1).

### Experimental Design and Sampling

Genotypes were grown in a randomized complete block design with three replications in the research farm of College of Agriculture



**Figure 1.** Inulin pathway has two phases (right to left), before and during cold nights. At the first phase, two molecules of sucrose (a) transformed to 1-kestose (c) and glucose (b) by *1-SST* enzyme. *1-FFT* enzyme transfers a fructosyl unit from donor to receiver inulin which leads to production of inulin with elongated fructose chain (d), totally. At the second phase, due to cold nights at the end of growing season, *1-FEH* gene is expressed and *1-SST* down regulated. Low *DP* inulin (e) produced with removing fructosyl unit (f) from end of chain by *1-FEH* enzymes. Subsequently, other functions of *1-FFT* appear and produce 1-kestose using sucrose and free fructosyl units or inulo-n-ose (g) by applying free fructosyl units.

**Table 1.** Genotypes used in this experiment.

Cultivar	Variety/Place of gathering	Place of gathering
Root chicory (Root type)	Orchies	Sugar beet Ins., Iran
Iranian genotype (Wild type)	Zanjan, Iran	Sugar beet Ins., Iran



and Natural Resources, University of Tehran, Iran (Latitude: 35.480783 and Longitude: 50.574147, with 280 mm average rainfall and 1,290 m of elevation from sea level) at the end of April, 2013. Each plot had five rows with 0.5 m distance and 2 m length with plant density of 8 plants per square meter. No fertilizer was applied and weeds were controlled by hand-pulling method for 3 times during cultivation. Roots were randomly sampled three times (60, 90, and 120 days after planting).

### Analysis of Relative Gene Expression Using Quantitative Real-Time-PCR

Total RNAs were extracted from 100 mg of 5 roots at three different times with 3 biological replicates for each genotype (Zanjan Indrace and Orchies) using the TRIOZOL reagent (Dena Zist Kit). Then, cDNAs were synthesised by the First Strand cDNA Synthesis Kit (Bioer) according to manufacturer's protocol. Specific primers were designed for qRT-PCR as shown in Table 2. Real-time quantitative PCR was performed by Rotor gene-Q set (Qiagene) using SYBR I Green in a 10  $\mu$ L reaction volume containing 4  $\mu$ L of SYBR (Bio Pars, GUASNR), 0.5  $\mu$ L of each forward and reverse primer of each gene (10 pmol), 4  $\mu$ L of diluted cDNA template, and sterile double-distilled water. The thermo amplification conditions were: 1 minute at 94°C for denaturation; 40 cycles of 30 seconds at 94°C, 20 seconds at 59°C, and 20 seconds at 72°C. After acquisition of data (Ct or threshold cycle value), relative genes expression levels were calculated by Relative Expression Software Tool (REST) V.2.0.13 (Pfaffl et al., 2009) using *Actin* as internal control. The expression

ratios for landrace genotype as sample were calculated relative to the expression in Orchieas cultivar as control [Equation (1)].

$$\text{Relative expression of interested gene} = (E_{\text{target}})^{\Delta CP_{\text{target}}} / (E_{\text{ref}})^{\Delta CP_{\text{ref}}} \quad (1)$$

Where,  $E$  is efficiency of real time PCR for target gene ( $E_{\text{target}}$ ) and reference gene ( $E_{\text{ref}}$ )

Measurement of Inulin, 1-kestose, Fructose, Glucose, Sucrose and DP of Inulin

Total carbohydrate was extracted by pooling and chopping of roots of each genotype. First, 10 g of chopped roots in a flask were added to 120 cc of distilled water (1 to 12 ratio). This mixture was kept for 40 minutes on a water bath (80°C). Samples were then filtered through a Whatman no. 1 filter round paper to obtain Chicory Root Extract (CRE). HPLC-ELSD (Evaporative Light-Scattering Detection) with Bio-Rad Aminex-HPX-87P column was used to detect fructose, glucose, and sucrose concentration on the basis of retention times and standard curves. Also, total fructose and glucose concentrations were determined after acid hydrolysis of inulin by adding 5 mL of sulfuric acid 98% to 1 mL of CRE in order to calculate average DP of inulin on the basis of Equation (2). Ion exchange chromatography with Aminex-HPX-87H column was used to measure inulin and 1-kestose contents.

$$DP (\text{degree of polymerization}) = \frac{*fructose \%}{*glucose \%} + 1 \quad (2)$$

Where, \* denotes concentration of fructose and glucose after acid hydrolysis.

### Statistical Analysis

All statistical analysis was performed by

**Table 2.** Primers of the studied genes for inulin biosynthesis.

Gene	Forward primer	Reverse primer	Melting temperature	Product size
<i>1-SST</i>	GTGGGTCGGCGTTTATCCAT	ATCTCCGCCCTGATGGTTGT	58	154
<i>1-FFT</i>	AATGTTGGAGGAGCCGTTGC	CGCGATATCAAGTGCGCTGT	58	191
<i>1-FEH I</i>	AGCATCTACGGAGCTTTTCTTG	CTTCTCCAAAACCTTTCGAC	55	147
<i>1-FEH II</i>	CCCGACCCAAGAAGCTGACA	CTTTGAATCGCTGCCGGTGT	59	185
<i>ACTIN</i>	CTCGAAACCGCCAAATCCAG	CTTTCGGCTCCGATGGTGAT	59	117

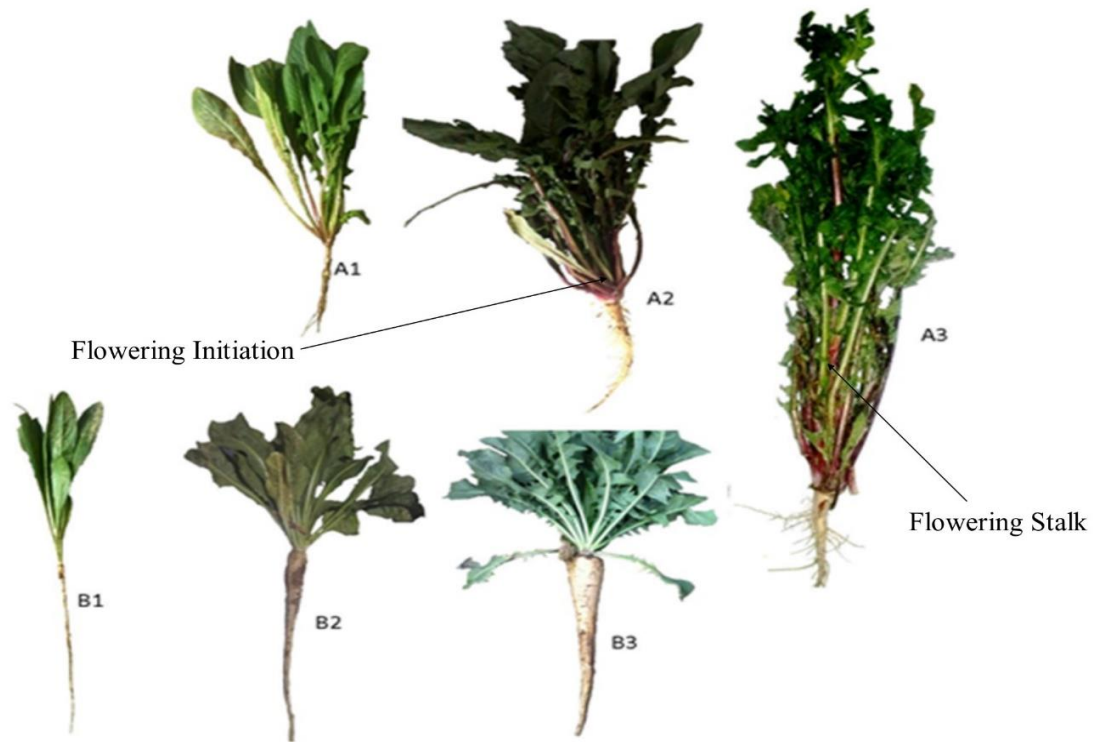
SAS9.1.3 software and the data is presented as mean±SE (SAS, 1992).

## RESULTS

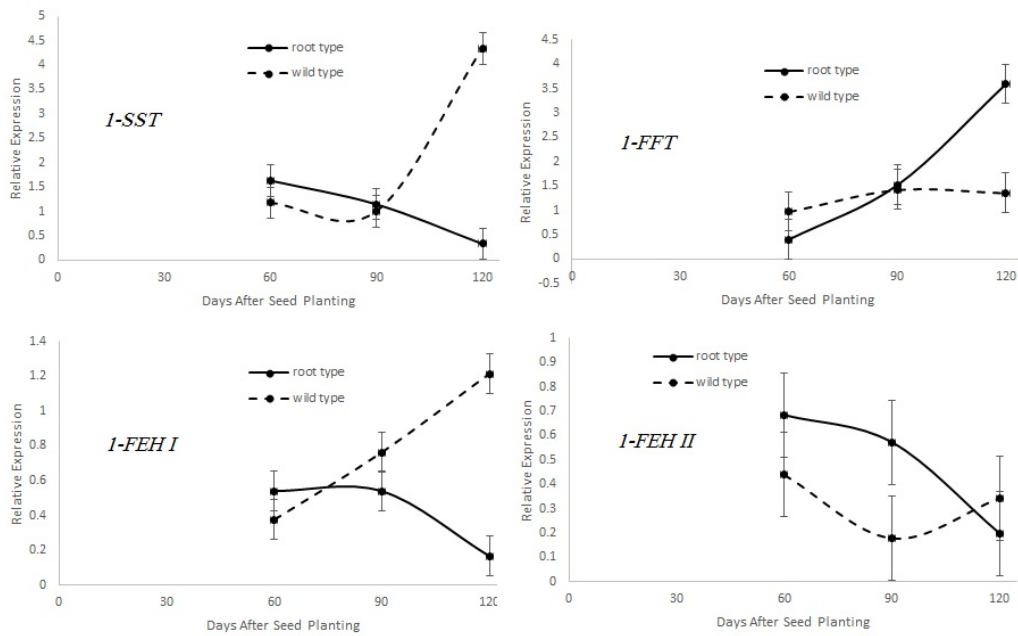
Although relative expression of *1-SST* as the first gene involved in inulin biosynthesis in Iranian genotypes was lower than the level of *1-SST* in Orchies at the beginning of normal phase, its expression was emulated at the end of growing season (Figure 3). Higher expression of *1-SST* in wild type genotype occurred at the end of normal phase of inulin biosynthesis at flowering stage, 90 days after seed planting (Figures 2 and 3). Glucose concentration, as the main product of *1-SST* function, was descending in Orchies cultivar, while it was ascending in the wild genotype corresponding to *1-SST* (Table 3). On the other hand, expression of *1-FFT* as the complementary gene for inulin biosynthesis and inulin polymerization was much lower in the wild type genotype of Zanzan compared to Orchies cultivar when it passed flowering formation phase at 90 days after seed planting (Figures 2 and 3). While relative expression of *1-FFT* in the wild genotype was low and constant, it was high and upward in Orchies cultivar (Figure 3). Corresponding function of *1-FFT* was more obvious in Orchies cultivar and its inulin average DP increased up to 10 by the end of the normal phase of inulin pathway, but average DP of inulin in the wild genotype never increased higher than 4 (Table 3). High expression of the degrading gene, *1-FEH I*, in the wild type genotype happened during the growing season when flowering initiation occurred from 90 days after planting to the end of growing season (Figures 2 and 3). This demonstrates that *1-FEH I* was stimulated by factors other than cold nights, thereby concentration of the resultant fructose was higher in the wild chicory of Zanzan than Orchies cultivar (Figure 3). Unlike *1-FEH I*, *1-FEH II* was not induced by other factors than cold nights and its expression was low and constant in the wild and root type genotypes (Figure 3). Inulin content with high DP rank as the final

**Table 3.** Saccharides values of inulin pathway in landrace genotype and Orchies cultivar.

Genotype	Days after planting	Saccharide						Average DP
		Inulin (DP>2) (mg g <sup>-1</sup> of fresh root)	1-Kestose (mg g <sup>-1</sup> of fresh root)	Sucrose (mg g <sup>-1</sup> of fresh root)	Glucose (mg g <sup>-1</sup> of fresh root)	Fructose (mg g <sup>-1</sup> of fresh root)		
Orchies	60	134.40±9.86	5.54±0.57	33.40±3.36	50.46±6.82	5.22±0.72	6.6	
	90	170.80±6.42	7.08±0.83	28.03±3.32	24.20±3.03	8.56±1.03	8.1	
	120	201.00±11.37	3.58±0.46	26.80±0.84	14.86±2.28	10.16±1.63	10.2	
Zanzan wild type	60	64.80±4.21	3.54±0.72	20.12±1.22	13.00±2.01	4.74±0.61	3.5	
	90	69.60±10.36	4.82±0.79	28.02±2.23	23.84±2.46	10.82±0.79	4.1	
	120	78.62±7.95	2.46±0.91	25.41±2.70	37.14±3.91	16.92±0.87	3.3	



**Figure 2.** Three times of root sampling (60, 90, and 120 days after seed planting) for Iranian genotype (A1 to A3) and Orchies cultivar (B1 to B3).



**Figure 3.** Trend of relative expression of inulin pathway genes in the wild and root type chicory during growing season.

product of inulin pathway was much higher in the root type genotype than wild type (Table 3) because of the efficient expression of inulin pathway genes particularly *1-FFT* for inulin biosynthesis in Orchies cultivar and adverse effect of degrading enzyme *1-FEH I* in the wild type genotype (Figure 3). So, it can be understood that fluctuation in expression levels of inulin pathway genes, except *1-FEH II*, were strongly related to flowering morphogenesis of Zanzan wild type genotype of chicory.

## DISCUSSION

Although *1-FEH* enzyme plays its part, it is by no means the only cause of inulin quality and quantity reduction in chicory (Van Den Ende *et al.*, 1996a; De Roover *et al.*, 2000; Van Arkel *et al.*, 2012). Spraying sucrose was recommended to stimulate inulin biosynthesis by many researchers (Baert and van Bockstaele, 1993; Baert 1997; Améziane, 1997). Storing of inulin in the Orchies cultivar genotype started earlier and stronger by producing high sucrose content at 60 days after seed planting (Table 3) and higher expression of *1-SST* gene (Table 3) for converting sucrose to 1-kestose and glucose (Figure 3). Good correlation between concentrations of free glucose (one of the products of 1-SST) in roots with expression level of *1-SST* gene was approved by Van Den Ende and Van laere (1996). High glucose content at 60 days after planting in Orchies cultivar demonstrates *1-SST* activity at the beginning of normal phase for inulin pathway, which declined by the end of that phase (Figure 3). Unlike Orchies cultivar, expression of *1-SST* gene was ascending for wild type genotype due to emerging flowering stalk in the first year (bolting) which initiated at 90 days after planting (Figure 2). Fructan 1-ExoHydrolase (*1-FEH*) enzymes such as invertases, which release fructose from sucrose, produce fructose from the end of inulin fructose chain to supply reproductive

cycle requirement. (Van den Ende *et al.*, 2002; Verhaest, 2005). Bolting hampered inulin biosynthesis in the wild type genotype and caused expression of inulin degrading gene, *1-FEH I*, to provide the required carbohydrate and energy for flowering (Shoorideh *et al.*, 2016). Low expression of *1-FEH II* in both wild and root type genotypes (Figure 3) indicates that it was not induced by factors other than cold nights. Maroufi *et al.* (2012), Michiles *et al.* (2004) and Van den Ende *et al.* (2001) also demonstrated that *1-FEH II* was induced by cold treatments and *1-FEH I* was stimulated during storage periods of roots. Consequently, expression of *1-FFT* gene for elongation of inulin fructose chain in the wild type genotype was repressed by flowering initiation and, hence, average *DP* of that genotype was much lower than Orchies cultivar and concentration of free fructosyl units in bolted genotype was higher than Orchies cultivar (Table 3). Expression of *1-FEH* genes in Orchies cultivar was postponed to cold nights in October, not for flowering stalk but for overwintering (Pilon-Smits *et al.*, 1995). RNAi technique for silencing of *1-FEH I* and *II* genes are recommended by some researchers to avoid inulin degradation during autumn and storing of roots and obtaining more inulin with higher *DP* (Kusaba, 2004).

Generally, high expression of *1-SST* at the beginning of normal phase for inulin biosynthesis in Orchies was an effective factor on inulin storing in root with high quality, but declining of *1-SST* expression through normal phase restricted the amount of inulin storing and quality. Expression trend for *1-SST* in the wild genotype was the opposite. Low expression of *1-SST* in the wild type limited initiation of inulin biosynthesis, but continually high expression of *1-SST* would be a promising case. Genetic engineers tried to expand activity of *1-SST* in root chicory genotypes by its overexpression using constitutive promoters or applying *1-SST* from other resources such as *Taraxacum officinale* (Maroufi *et al.*,



2016). Meanwhile, it can be obtained by classical breeding using such wild genotypes through some classical breeding methods. Therefore, measuring sequential expression of *I-SST* gene during normal phase of inulin biosynthesis in various wild types of chicory genotypes can be helpful for detecting the more promising genotypes in breeding program of industrial root type chicory.

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## بیان فاصله ای ژنهای مسیر بیوسنتز اینولین در کاسنی وحشی و ریشه ای

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### چکیده

کاسنی (*Cichorium intybus* L.) بعنوان یک گیاه شاخص مدیترانه‌ای، در سرتاسر جهان با مصارف تجاری متفاوتی مثل سالادی، علوفه‌ای، منبع تولید اینولین و جایگزین قهوه پخش شده است.



اینولین بعنوان یک ترکیب پری بیوتیک دارای خاصیت ارتقاء دهنده گی سلامت است که همین موضوع منجر به آشکار شدن مسیر بیوسنتز آن شده است. در فاز نرمال و ره رشد رویشی دو آنزیم ۱-SST و ۱-FFT در سنتز اینولین دخیل هستند. آنزیم‌های ۱-FEH-اینولین را تحت تاثیر وقوع شب‌های سرد و یا دیگر فاکتورهای محیطی به واحدهای فروکتوسیل تجزیه می‌کند. جهت مقایسه شدت عملکرد این ژن‌ها در ژنوتیپ وحشی نسبت به رقم زراعی کاسنی ریشه‌ای اریس در سه مرحله ۶۰، ۹۰ و ۱۲۰ روز پس از کاشت بدر، بیان نسبی ژن‌ها همراه با متابولیت‌های مربوطه آنها با استفاده از تکنیک‌های RT-qPCR و HPLC اندازه‌گیری شدند. نتایج بیان نشان داد که برخلاف رقم اریس، بیان نسبی 1-SST در ژنوتیپ وحشی صعودی است، بیان ۱-FFT خیلی کم و ثابت است و سطوح بالایی از بیان ژن ۱-FEH- I طی فصل رشد در نتیجه انگیزش گل‌دهی رخ داده است. همچنین غلظت‌های فروکتوز و گلوکز بترتیب در نتیجه فعالیت ژن‌های ۱-SST و 1-FEH I در ژنوتیپ وحشی صعودی بودند. درجه پلی‌میرزاسیون (DP) اینولین تولید شده تقریباً هیچ افزایشی در نتیجه عملکرد ضعیف ۱-FFT در ژنوتیپ وحشی (کمتر از ۵) نداشت اما DP اینولین تولید شده توسط رقم اریس بالاتر از ۱۰ بود، علی‌رغم اینکه بیان ۱-SST طی فصل رشد کاهشی بود. بنابراین امکان کارآمدتر کردن مسیر بیوسنتز اینولین در کاسنی ریشه‌ای اریس توسط بسط دوره بیان و افزایش میزان بیان ۱-SST با استفاده از منابع کاسنی وحشی از طریق اصلاح کلاسیک آمیزش برگشتی و یا روش‌های بیوتکنولوژی وجود دارد.