

**Differential expression of fructan 1-exohydrolase genes involved in inulin biodegradation in chicory (*Cichorium intybus*) cultivars**Asad Maroufi<sup>1\*</sup>, Erik Van Bockstaele<sup>2,3</sup> and Marc De Loose<sup>4,5</sup><sup>1</sup>Department of Agronomy and Plant Breeding, Faculty of Agriculture, University of Kurdistan, Sanandaj, Iran<sup>2</sup>Department of Plant Production, Faculty of Bioscience Engineering, Ghent University, Coupure Links 653, 9000 Ghent, Belgium<sup>3</sup>Institute for Agricultural and Fisheries Research (ILVO), Plant Sciences Unit, Caritasstraat 21, 9090 Melle, Belgium<sup>4</sup>Department of Plant Biotechnology and Genetics, Faculty of Sciences, Ghent University, K.L. Ledeganckstraat 35, 9000 Ghent, Belgium<sup>5</sup>Institute for Agricultural and Fisheries Research (ILVO), Burg Van Gansberghelaan 115 bus 1, 9820 Merelbeke, Belgium

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**Abstract**

Fructan 1-exohydrolase (1-FEH; FEH) enzymes are involved in inulin degradation in the roots of chicory. Higher *FEH* expression in cold temperatures can decrease the quality and the quantity of the inulin. This is the case at the end of the growing season and during cold storage. Little is known at molecular level whether the expression levels of fructan 1-exohydrolase genes vary among chicory cultivars, especially during cold storage of roots. Real-time RT-PCR is the most sensitive method for the detection of low abundance mRNAs. Quantitative real time PCR (RT-qPCR) analysis of the *FEH* mRNAs (including *FEHI* and *FEHII*) in cold stored roots of three chicory cultivars was studied. RT-qPCR results showed variable levels of *FEHII* and *FEHI* expression in different phenotypes of the chicory cultivars studied. Moreover, longer cold storage of roots significantly induced expression levels of *FEHII* in some chicory phenotypes. These results could be used in breeding programmes to increase inulin content in root chicory by selecting genotypes based on their *FEH* expression profiles.

**Keywords:** root chicory, inulin degradation, breeding, cold storage, quantitative real time PCR.**Abbreviations:** 1-FEH:(FEH), fructan 1-exohydrolase; *ACT*, actin; Cp, crossing cycle number; CV, coefficient of variation; DP, degree of polymerisation; *EF*, elongation factor; *M*, average gene expression stability; NTC, no-template control; RT-qPCR, quantitative real time PCR.**Introduction**

Inulin-type fructan is mostly found in the *Asteraceae* family, to which chicory belongs. Root chicory is considered one of the most important crops for the commercial extraction of inulin on an industrial scale (Franck and De Leenheer, 2005). Plant source, harvest date and storage length are the most important factors affecting the degree of polymerisation (DP) of inulin (Beirao-da-Costa, 2005). Recovery of inulin from chicory roots is typified by relatively poor yields and the inulin often loses quality due to endogenous degradation during and after harvest (Banguela and Hernandez, 2006). Inulin production is hampered by the induction of *FEH* activity, which affects fructose production in field-grown chicory roots during autumn (Van den Ende and Van Laere, 1996; Van den Ende et al., 1996a). The *FEH* activity increases when the temperature drops, which leads to an increasing amount of free fructose (Van den Ende and Van Laere, 1996). Induction of the fructan 1-exohydrolase gene expression reduces the total inulin amount and its mean degree of polymerisation (DP). This breakdown of inulin significantly reduces inulin yield and its usefulness for industrial applications. When striving to improve root chicory cultivars to increase the amount of inulin with high DP, fructan degradation by induction of *FEH* has to be

avoided. Therefore, reduction of *FEH* activity may extend the harvest period of chicory plants without risking inulin breakdown. Better understanding of the molecular regulation of *FEH* expression in root type chicory genotypes during cold storage may also provide important information and tools necessary to increase inulin yield. Recent molecular studies of chicory have led to the identification and characterisation of fructan 1-exohydrolase genes (Van den Ende et al., 2000; Van den Ende, 2001; Michiels et al., 2004). In chicory, fructan 1-exohydrolase (1-FEH; FEH), which is involved in the degradation of inulin, consists of three enzymes including 1-FEHIIa (FEHIIa), 1-FEHIIb (FEHIIb), and 1-FEHI (FEHI). Available sequences of the related genes allow a comprehensive study of their expression. The abundance of mRNA is often used as the direct measure of gene expression. Analysis of gene expression has become increasingly important for understanding how genetic profiles affect the function and phenotype of different cells and tissues. Moreover, in both animals and plants, more attention has been paid to gene expression in specific tissues at certain developmental stages and with specific phenotypes. A recently developed reverse transcriptase-quantitative PCR is one of the most rapid and quantitative methods used to

determine the level of the target transcripts. RT-qPCR is effective in characterising the expression patterns of mRNA of particular genes, even from little sample material (Wang and Brown, 1999). Application of RT-qPCR has become very significant in gene expression studies in plants (Gachon, 2005) but has only been used in a few studies in chicory (Legrand et al., 2007). Therefore, we used RT-qPCR method to evaluate the expression levels of *FEHI* and *FEHII* in different genotypes of chicory.

## Results

### Evaluation of expression ratios of reference genes

With RT-qPCR, the levels of a specific transcript can be determined relative to the levels of an internal control RNA. This reference is usually a stably expressed housekeeping gene. The *EF* and *ACT* were selected as stable reference genes in leaf and root tissues in chicory (Maroufi et al., 2010). The expression pattern of *ACT* relative to *EF* illustrates the relative stable expression ratio of these two genes across root tissues in all samples at the experimental condition (Fig. 2). The reference genes showed M (average gene expression stability) value lower than 0.5. geNorm recommends using an M value below the threshold of 1.5 to identify (sets of) reference genes with stable expression (geNorm manual). Therefore, according to geNorm they are stably high expressed and can surely be used as reference genes to quantify the level of target gene(s) with RT-qPCR.

### Relative expression level of *FEHI* in cold conditions

The expression level of *FEHI* in the roots of two industrial chicory cultivars, a cloned plant, and a “*Witloof*” chicory cultivar (Table 1) were assessed during their time in cold storage. RT-PCR for all samples was performed in 384-well plates in a Lightcycler480 and Cp values were collected. The Cp values were transformed mathematically by applying the specific amplification efficiency method to relative expression levels according to the qBase manual (Hellemans et al., 2007). A relative quantification of the expression of *FEHI* was achieved by calibrating the expression of *FEHI* with the reference genes. The *FEHI* expression pattern for all roots in different times is presented in Fig. 3a. Two RNAs from each root of the cloned plant were isolated to check the technical variation in the experiment. Two independent RNA samples from roots of the cloned plant exhibited nearly the same *FEHI* expression level (Fig. 3a). It can therefore be concluded that RNA extraction did not add additional variation to the level of gene expression. Roots from all cultivars showed different expression level after five weeks storage time (indicated with blue bars; Fig. 3b), but they were not significantly different (Fig. 3b). Roots belong to ‘Dolce’ exhibited higher variation than the other cultivars’ roots (Table.3; Fig.3b). Only roots of the cloned plant after nine weeks of cold storage (red bars) showed significantly higher *FEHI* expression level than the same roots stored for five weeks (Fig. 3b; Table 3). However, roots from ‘Dolce’, ‘Hera’ and ‘Vintor’ did not show significant up-regulation for *FEHI* when roots were stored for a longer time (Fig. 3b; Table 3). In addition, for three successive RNA samples taken for ‘Vintor’ roots, *FEHI* did not show up-regulation in the function of cold storage time (Fig.3c).

### Relative expression level of *FEHII* in cold conditions

The *FEHII* expression level for two industrial root chicory cultivars, a cloned plant and a “*Witloof*” chicory cultivar were

also studied in the cold stored roots. As the nucleotide sequences of *FEHIIa* and *FEHIIb* cDNAs are very homologous (94% identity at the DNA level), no specific primers could be designed to differentiate between the two isoforms. Therefore, we designed a primer pair to amplify both *FEHIIa* and *FEHIIb* (Fig.1). The selected amplicons were confirmed by sequencing RT-PCR products. Accordingly, the designed primer pairs can amplify both amplicons belong either to *FEHIIa* or *FEHIIb*. A relative quantification of the expression of *FEHII* was achieved by calibrating the expression of *FEHII* with the reference genes. Fig. 4a shows the expression levels of *FEHII* in the cold stored roots at two time points. After five weeks cold storage of roots (blue bars), individual chicory roots belong to ‘Hera’ and ‘Dolce’ cultivars exhibited variation for the level of *FEHII* expression (Fig. 4a). However, the differences between the two cultivars were not considerable (Fig. 4b). Additionally, higher expression levels were observed for the roots of ‘Vintor’ (Fig. 4b; Table 4). After nine weeks of cold storage of roots (red bars) all cultivars or the cloned plant showed different *FEHII* expression levels (Fig. 4a; Table 4). After nine weeks of root storage (red bars) compared to five weeks (blue bars), all cultivars or the cloned plant showed considerably higher expression levels of *FEHII* except for the cultivar ‘Hera’ (Fig. 4b). But different phenotypes from the same cultivar showed different induction of *FEHII* in longer cold storage of roots. Higher *FEHII* expression level indicates significant up- regulation of gene expression at longer storage time in some genotypes such as the cloned plant, ‘Dolce’, and ‘Vintor’. In addition, the expression pattern of *FEHII* in ‘Vintor’ at three time periods (harvest time, after five weeks of root storage, and after nine weeks of root storage) clearly showed a significant increase of *FEHII* expression in the function of root storage time under cold conditions (Fig. 4c), while it was not observed for *FEHI* (Fig. 3c).

## Discussion

It is known that the yield of inulin at harvest time and during storage is decreased by a group of enzymes called fructan 1-exohydrolase (1-FEH: FEH) (Van den Ende and Van Laere, 1996; Van den Ende et al., 2001; Michiels et al., 2004 ). FEH enzymes are responsible for inulin depolymerisation in roots of chicory (Van den Ende et al., 2000; Van den Ende et al., 2001). In a cold environment (3 weeks at 4°C), inulin clearly degrades in roots of chicory, and results in lower-DP inulin and mainly fructose (Franck and De Leenheer, 2005). Consequently, there is a correlation between FEH activity and inulin content (Van den Ende and Van Laere, 1996; Van den Ende et al., 1996). We have studied the expression level of the genes encoding the two enzymes (FEHI and FEHII) in function of root storage time under cold conditions for different chicory cultivars with a different genetic background. RT-qPCR, a very accurate and sensitive method, was used to measure the expression level of *FEH* genes. In previous studies, the *FEH* expression was studied using Northern Blotting and only for a “*Witloof*” chicory cultivar (Van den Ende et al., 2000; Van den Ende et al., 2001; Michiels et al., 2004). Gene expression studies by Northern Blot rely on having access to relatively large amounts of material either in the form of RNA or the cells from which it is to be isolated. Therefore, the small changes in the amount of transcripts would not be detected. In this study using RT-qPCR, new insight into the expression level of *FEH* was obtained. We observed that the levels of *FEHI* are substantially lower than the levels of *FEHII*. The expression levels of *FEHI* are not significantly different for root chicory

**Table 1.** The cultivars and RNA extraction at different time points.

Plant material (group)	RNA extracted (fresh)	RNA extracted after 5 weeks of root storage at 4°C	RNA extracted after 9 weeks of root storage at 4°C
Cloned plant (C)	-	+	+
'Hera' (H)	-	+	+
'Dolce' (D)	-	+	+
'Vintor' (V)	+	+	+

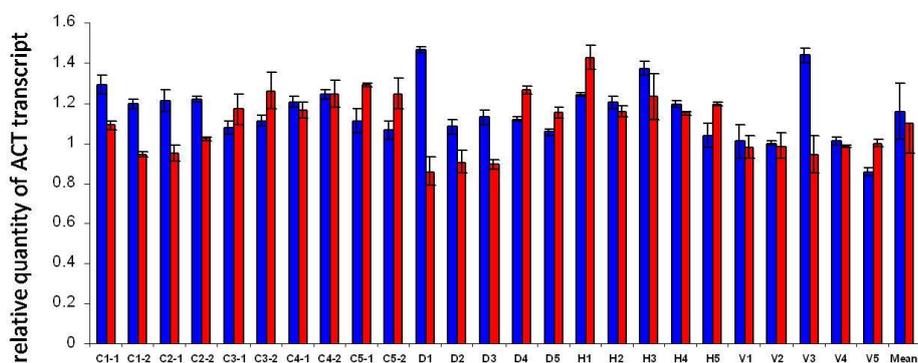
  

FEHIIa	4417	TAAAGACTTGAAAGAACAAAGTGCAATCTTTTTTAGGGTTTTTCAAACCAACTTGGACG	4476
FEHIIb	1356	TAAAGACTTGAAAGAACAAAGTGCAATCTTTTTTAGGGTTTTTCAAACCAACTTGGGCG	1415
FEHIIa	4477	ATACTCTGTGCTCATGTGTAGTGATCTTAGCAGGTCTACTGTTAGAAGCAATATCGACAC	4536
FEHIIb	1416	ATATTCTGTTCTCATGTGTAGTGATCTTAGCAGGTCTACCGTTAGGAGCAACATCGACAC	1475
FEHIIa	4537	GACAAGTTATGGTGC	4551
FEHIIb	1476	GACAAGTTATGGTGC	1490

**Fig 1.** Alignment of *FEHIIa* and *FEHIIb*. The two amplicons have high sequence similarity. Black arrows indicate the designed prime pairs. The numbers indicate the reference base pairs, bases 1 to 5862 for *FEHIIa*; GenBank accession number AY323935.

**Table 2.** Designed primer pairs for reference genes (*EF* and *ACT*), *FEHII*, and *FEHI* for RT-qPCR.

Gene	GenBank accession number	Primer sequences (forward)	Primer sequences (reverse)
<i>ACT</i>	EF528575	CCAAATCCAGTCATCAGTCG	TCTTTCGGCTCCGATGGTGAT
<i>EF</i>	AY378166	CATGCGTCAGACGGTTGCTGT	CTTCACTCCCTTCTTGCTGC
<i>FEHII</i>	AY323935	TAAAGACTTGAAAGAACAAAGTG	GCACCATAACTTGTCTGTGTCG
<i>FEHI</i>	AJ242538.1	AGCATCTACGGAGCTTTTCTTG	CTTCTCCACAAAACCTTTCGAC



**Fig 2.** Expression ratio of *ACT* against *EF* at two time points after cold storage of roots in all samples. Blue bars indicate expression level in the RNA extracted after five weeks of root storage and red bars indicate the RNA extracted after nine weeks of root storage. The 'Vintor' 2 (V2) sample in expression profile after five weeks of root storage is set as calibrator in qBase analysis. Error bars indicate standard error of the mean. Letters are used to describe cultivars, i.e., c=cloned plants; D='Dolce'; H='Hera'; V='Vintor'; the first digit indicates the number of the root and the number after the dash indicates the number of RNA extracted.

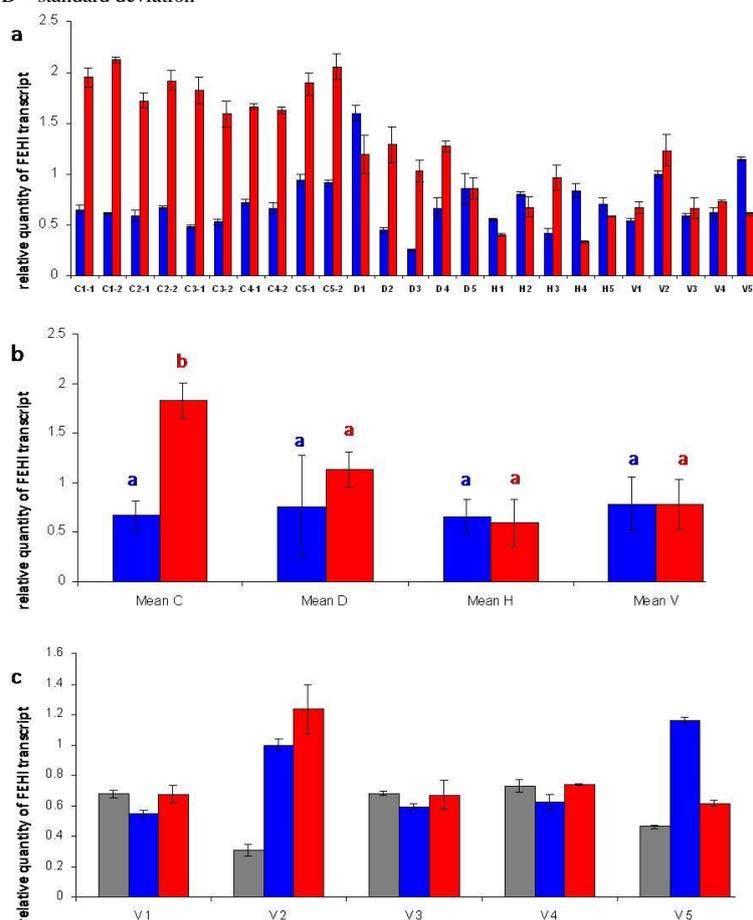
in comparison with "Witloof" when testing gene expression at two time periods. Furthermore, the induction factor for *FEHI* expression (Table 3) during cold storage shows no significant increase in the roots of 'Hera' and 'Vintor'. The induction factor for 'Dolce' was 0.48, which is not a significant increase (Table 3). In contrast, the induction of *FEHII* upon root cold storage for all cultivars clearly increased. The lowest induction was for Hera's roots and the highest induction was for Dolce's roots (Table 4). Additionally, high expression levels and also a substantial induction for *FEHII* are observed for all roots belonging to the cultivar 'Vintor'. This indicates the role of cold storage in the induction of *FEHII* expression in the chicory roots. 'Vintor' belongs to 'Witloof' chicory, which is used for the production of Belgian endive. It is known that for the production of 'Witloof',

during root storage, the inulin slowly breaks down and leads to an increase in the soluble sugar fraction. This process is necessary for growth and development of chicons (a small white head of leaves ringed with regions of yellow-green) during the forcing stage (Corey et al., 1990). Therefore, it would be reasonable that the expression level of *FEHII* in the roots belonging to this cultivar increases greatly during root storage under cold conditions. Chicory root cultivars are normally a synthetic variety. Genetic variation of individual phenotypes (roots) does exist in synthetic variety, therefore, this might be a possible reason for *FEHI* and *FEHII* variation among roots belonging to the same cultivar. Clearly, root cultivars have been selected from 'Witloof' genotypes for better root shape, higher root yield and inulin content. Eventually, in the selection breeding programmes the

**Table 3.** Mean expression level of *FEHI*.

Plant material <sup>1</sup> (group)	Mean <i>FEHI</i> expression after 5 weeks of root storage (a)	SD <sup>2</sup>	Mean <i>FEHI</i> expression after 9 weeks of root storage (b)	SD	Induction factor = (b-a)/a
cloned plant	0.67	0.14	1.83	0.18	1.70
'Dolce'	0.76	0.51	1.13	0.18	0.48
'Hera'	0.66	0.17	0.59	0.24	-0.1
'Vintor'	0.78	0.27	0.78	0.25	0

<sup>1</sup>Five roots per cultivar; <sup>2</sup>SD = standard deviation



**Fig 3.** a) Relative quantities of the FEHI transcript in roots of three cultivars and a clone plant after root storage for five weeks (blue bars), and root storage for nine weeks (red bars) b) Mean relative quantities of the FEHI transcript of three cultivars and a clone plant after root storage for five weeks (blue bars) and root storage for nine weeks (red bars). c) Relative quantities of the FEHI transcript in roots at three time points of RNA sampling for 'Vintor'; grey bars indicate RNA extracted for roots at harvest time; blue bars after five weeks of root storage; and red bars after nine weeks of root storage. The 'Vintor' 2 (V2) sample in expression profile after five weeks of root storage is set as calibrator in qBase analysis.. Error bars indicate standard error of the mean. Letters are used to describe the cultivars, i.e., c=cloned plants; D='Dolce'; H='Hera'; V='Vintor'; the first digit indicates the number of root and the number after the dash indicates the number of RNA extracted. Means followed by the same letter are not significantly different at  $P < 0.01$ .

genotypes showing lower level of *FEH* might have indirectly been selected. Consequently, by the expression analysis of *FEH* genes for any phenotype, there would be additional data to directly assess the level of *FEH* expression. This would be helpful for selection breeding aiming to improve inulin yield of root chicory.

## Materials and methods

### Plant material and fructan 1-exohydrolase genes

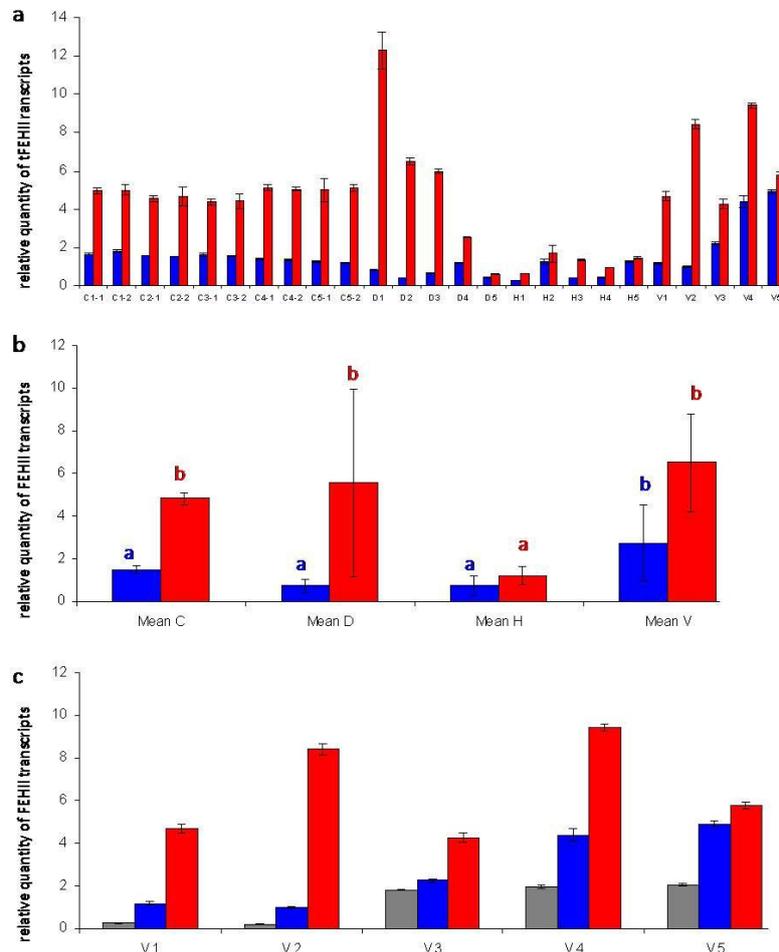
Plant material consisted of 1) one root type chicory cloned plant, 2) 'Hera' and 'Dolce' (two industrial chicory root cultivars), and 3) 'Vintor' (a "Witloof" chicory cultivar). These plants were grown in the field at the Institute for

Agricultural and Fisheries Research (ILVO) in Merelbeke, Belgium. 'Hera' is a cultivar bred for high root yield, high inulin content and high DP. 'Dolce' has also been bred for high DP quality, high inulin content, and to resist bolting (ILVO, Belgium). These two cultivars are genetically far from each other (Joost Baert, ILVO, personal communication). 'Vintor' is a "Witloof" chicory cultivar and has been bred for "Witloof" production (Nunhems, The Netherlands). The cloned plant was selected in a recurrent selection programme from polycross progenies of cultivar 'Hera' (Joost Baert, ILVO, personal communication). Seeds for the cultivars were directly sown in the field. The clone was made from root pieces of a selected plant. Root pieces

**Table 4.** Mean expression level of *FEHII*.

Plant material <sup>1</sup> (group)	Mean <i>FEHII</i> expression after 5 weeks of root storage (a)	SD <sup>2</sup>	Mean <i>FEHII</i> expression after 9 weeks of root storage (b)	SD	Induction factor = (b-a)/a
cloned plant	1.50	0.18	4.82	0.29	2.21
'Dolce'	0.73	0.32	5.57	4.4	6.63
'Hera'	0.73	0.49	1.21	0.41	0.65
'Vintor'	2.74	1.81	6.52	2.29	1.37

<sup>1</sup>Five roots per cultivar; <sup>2</sup>SD = standard deviation



**Fig 4.-** a) Relative quantities of the FEHII transcripts in roots of three cultivars and a clone plant after root storage for five weeks (blue bars) and root storage for nine weeks (red bars). b) Mean relative quantities of the FEHII transcripts of three cultivars and a clone plant after root storage for five weeks (blue bars) and root storage for nine weeks (red bars). c) Relative quantities of the FEHII transcripts in roots at three time points of RNA sampling for 'Vintor'; grey bars indicate RNA extracted for roots at harvest time; blue bars after five weeks of root storage; and red bars after nine weeks of root storage. The 'Vintor' 2 (V2) sample in expression profile after five weeks of root storage is set as calibrator in qBase analysis. Error bars indicate standard error of the mean. Letter used to describe cultivars, i.e., c=cloned plants; D='Dolce'; H='Hera'; V='Vintor'; the first digit indicates the number of root and the number after the dash indicates the number of RNA extracted. Means followed by the same letter are not significantly different at  $P < 0.01$ .

were grown in vermiculite in the greenhouse to give rise to young plants. They were then transferred to the field. Five plants of each cultivar and the cloned plant were sampled at maturity in October. Each plant's root was carefully washed and used for further experiments. An RNA sample from 'Vintor' plants only was extracted from each selected root at harvest time (Table.1). Roots were then stored in a cold room (4 °C) for five weeks to extract RNA samples. In order to see the effect of longer cold storage on *FEH* induction, the same roots were stored in the cold room for four additional weeks before extracting another RNA sample (Table.1). Fructan (inulin) breakdown in chicory is catalysed by fructan exohydrolase (Van den Ende et al., 2000). FEHI and FEHII

are the enzymes known to be involved in inulin degradation in roots of chicory (Claessens et al., 1990; De Roover et al., 1999). 1-FEHII includes two independent enzymatically active isoforms that were termed FEHIIa and FEHIIb (Van den Ende et al., 2001).

#### RNA preparation and cDNA synthesis

Total RNA was isolated using the RNeasy Plant Mini Kit (Qiagen) from tissue of the upper part under the crown of root according to the manufacturer's instructions. Two RNA extracts were independently collected from the roots of the cloned plant. Concentration and purity of RNA was

determined using a Nanodrop ND1000 spectrophotometer (Thermo SCIENTIFIC). Total RNA was treated with TURBO™ DNase (Ambion) according to the manufacturer's instructions to remove all genomic DNA contamination. cDNAs were synthesised using 700 ng of total RNA for all samples by the SuperScript® VILO™ cDNA Synthesis Kit (Invitrogen) following the manufacturer's instructions in a final volume of 20 µl. The final cDNA products were diluted 15-fold prior to use in RT-qPCR.

#### Sequencing of *FEHIIa/FEHIIb* amplicon

A primer pair which may amplify *FEHIIa* or *FEHIIb* was designed by Primer Express version 2.0.0 (Applied Biosystems; Fig.1). To sequence the selected amplicon, first a PCR reaction containing 300 nM of each primer, 2 U of *Pfu* DNA polymerase (Promega), 400 µM dNTPs (Invitrogen), and 100 ng of cDNA in a total volume of 50 µl, was performed with the following programme: 95°C for 2 min and 35 cycles at 94°C for 30 sec, 58°C for 30 sec, and 72°C for 30 sec. PCR products were purified using the Qiaquick PCR purification kit (Qiagen) based on the manufacturer's instructions. PCR products were cloned using the TOPO TA Cloning Kit (Invitrogen) according to the accompanying instruction manuals. Sequencing reactions were performed on 500 ng of a number of purified plasmids using 100 nM of either M13 forward or M13 reverse primer in separate reaction, 4 µl of Big Dye Mix (Big Dye Terminator v1.1 cycle sequencing kit; Applied Biosystems) and 2 µl of sequencing buffer in a total volume of 20 µl. The reactions were analysed on the ABI PRISM 3130 Genetic Analyzer (Applied Biosystems). Derived sequences were compared to the respective sequences in the Genbank using vector NTi software (Invitrogen).

#### Real-time RT-PCR

The sequences of the genes used in RT-qPCR experiment included *FEHII*, *FEHI*, actin (*ACT*) and the elongation factor (*EF*) obtained from Genbank. The primers were designed using Primer Express3 software (Invitrogen; Table.2). *ACT* and *EF* were selected as the best reference genes to use for relative gene expression analysis in leaf and root tissues of chicory (Maroufi et al., 2010). RT-PCR reactions were performed in 384-well plates in a Lightcycler480 (Roche). For each RT-PCR reaction, 2.5 µl cDNA from a 15-fold diluted cDNA template, 300 nM of each primer, and 1X LightCycler® 480 SYBR Green I Master (Roche) in a final volume of 10 µl were mixed. All RT-PCR reactions were carried out in duplicate for each cDNA sample. An equivalent amount of total RNA for each sample was included in RT-PCR as control for genomic DNA contamination. A no-template control (NTC) was also included in each run for each gene. The experiment was performed twice in independent runs. The thermal profile of the RT-PCR reactions was 95°C for 5 min activation and denaturation, followed by 45 cycles of 95°C for 10 sec, and 59°C for 10 sec for fluorescence acquisition after each cycle. Finally, a melting curve was generated by increasing temperature from 65°C to 95°C. The "crossing cycle number" (Cp) was automatically determined for each reaction by the LightCycler480 SW 1.5 software with default parameters for the second derivative method.

#### Analysis of the relative expression level of target genes

RT-qPCR was used to study changes in the transcript level of *FEHII* and *FEHI* as a result of cold induction in roots of different chicory plants at different times. Cp values of *FEHI*,

*FEHII* as target genes, with *ACT* and *EF* as reference genes for all samples, were collected and imported into the qBase software. Amplification efficiency for each gene was calculated. The Cp values were transformed mathematically by using the specific efficiency method into relative expression levels (qBase Analysis). The Cp values were transformed into relative quantities for *FEHII* and *FEHI* expression by normalising them with selected reference gene and specific amplification efficiency (qBase Analysis; Hellemans et al., 2007).

#### Conclusion

It is clear that fructan 1- exohydrolase enzymes are the main reason for decreasing inulin yield in roots of chicory during harvest and cold storage time. Therefore, evaluation of fructan 1- exohydrolase expression levels of individual phenotypes in selection programmes to improve inulin content in chicory roots may provide extra information to the breeders. Different phenotypes exhibited different expression levels for *FEHI* and *FEHII* under cold storage conditions. *FEHI* during cold storage of different roots showed nearly the same expression level and no induction during longer cold storage. However, *FEHII* showed higher expression levels than *FEHI* and also showed more induction in function of a longer storage time under cold conditions. Consequently, directly measuring the level of *FEHII* expression in cold stored roots may yield valuable data to improve inulin yields of root chicory.

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#### References

- Baert JRA, Van Bockstaele E (1993) Cultivation and breeding of root chicory for inulin production. *Ind Crop Prod* 1: 229-234
- Baert JRA, Notte C (2002) Breeding of industrial chicory. Warcoing, 29 August presentation
- Banguela A, Hernandez L (2006) Fructans: from natural sources to transgenic plants. *Biotechnol Apl* 23(3)
- Beirao-da-Costa ML, Filipa MLS, Leitao AE, Januario MI (2005) Characterisation of inulin from chicory and salsify cultivated in Portugal. In: 2nd Mercosur Congress on Chemical Engineering and 4nd Mercosur Congress on Process Systems Engineering, Rio de Janeiro, Brazil. pp 9 August 14th to 18th 2005. (edicao electronica, ISBN 85-7650-034-4)
- Corey KA, Marchant DJ, Whitney LF (1990) Witloof chicory: A new vegetable crop in the United States. p. 414-418. In: Janick J, Simon JE, (eds.), *Advances in new crops*. Timber Press, Portland, OR
- Franck A, De Leenheer L (2005) Inulin. In Steinbuechel A, Ki Rhee S (ed) *Polysaccharides and Polyamides in the Food Industry: Properties, Production, and Patents*, Wiley-VCH
- Gachon C, Mingam A, Charrier B (2005) Real-time PCR: what relevance to plant studies? *J Exp Bot* 55:1445-1454
- geNorm software manual update. <http://medgen.ugent.be/~jvdesomp/genorm/>.

- Hellemans J, Mortier G, De Paepe A, Speleman F, Vandesompele J (2007) qBase relative quantification framework and software for management and automated analysis of real-time quantitative PCR data. *Genome Biol* 8:R23
- Legrand S, Hendriks T, Hilbert JL, Quillet MC (2007) Characterization of expressed sequence tags obtained by SSH during somatic embryogenesis in *Cichorium intybus* L. *BMC Plant Bio* 7:27
- Maroufi A, Van Bockstaele E, De Loose M (2010) Validation of reference genes for gene expression analysis in chicory (*Cichorium intybus*) using quantitative real-time PCR. *BMC Mol Bio* 11:15
- Michiels A, Van Laere A, Van den Ende W, Tucker M (2004) Expression analysis of a chicory fructan 1-exohydrolase gene reveals complex regulation by cold. *J Exp Bot* 55:1325-1333
- Van den Ende W, Van Laere A (1996) Fructan synthesizing and degrading activities in chicory roots (*Cichorium intybus* L.) during growth, storage and forcing. *J Plant Physiol* 149:43-50
- Van den Ende W, Michiels A, De Roover J, Verhaert P, Van Laere A (2000) Cloning and functional analysis of chicory root fructan 1-exohydrolase I (1-FEHI): a vacuolar enzyme derived from a cell-wall invertase ancestor? Mass fingerprint of the 1-FEHI enzyme. *Plant J* 24:447-456
- Van den Ende W, Michiels A, Van Wontergem D, Clerens S, De Roover J, Van Laere A (2001) Defoliation induces 1-FEHII (fructan 1-exohydrolase II) in witloof chicory roots. Cloning and purification of two isoforms (1-FEHIIa and 1-FEHIIb). Mass fingerprint of the 1-FEHII enzymes. *Plant Physiol* 126:1186-1195
- Wang T, Brown MJ (1999) mRNA quantification by real time TaqMan polymerase chain reaction: validation and comparison with RNase protection. *Anal Biochem* 269:198-201