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ORIGINAL PAPER

Mutations in chicory FEH genes are statistically associated with enhanced resistance to post-harvest inulin depolymerization

Nicolas Dauchot · Pierre Raulier · Olivier Maudoux · Christine Notté · Pierre Bertin · Xavier Draye · Pierre Van Cutsem

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Abstract
Key message **Nucleotidic polymorphisms were identified in fructan exohydrolases genes which are statistically associated with enhanced susceptibility to post-harvest inulin depolymerization.**
Abstract Industrial chicory (*Cichorium intybus* L.) root is the main commercial source of inulin, a linear fructose polymer used as dietary fiber. Post-harvest, inulin is depolymerized into fructose which drastically increases processing cost. To identify genetic variations associated with enhanced susceptibility to post-harvest inulin depolymerization and related free sugars content increase, we used a candidate-gene approach focused on inulin and sucrose synthesis and degradation genes, all members of the family 32 of glycoside hydrolases (GH32). Polymorphism in these genes was first investigated by carrying out EcoTILLING on two groups of chicory breeding lines exhibiting contrasted response to post-harvest inulin depolymerization. This allowed the identification of polymorphisms significantly associated with depolymerization in three fructan exohydrolase genes (FEH). This association was confirmed on a wider panel of 116 unrelated families in which the FEH polymorphism explained 35 % of the post-harvest variance for inulin content, 36 % of variance for sucrose content, 18 % for inulin degree of polymerization, 23 % for free fructose content and 22 % for free glucose content. These polymorphisms were associated with significant post-harvest changes of inulin content, inulin chain length and free sugars content.

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N. Dauchot (✉) · P. Van Cutsem
Research Unit in Plant Biology, University of Namur, 61 rue de Bruxelles, 5000 Namur, Belgium
e-mail: nicolas.dauchot@unamur.be

P. Raulier · P. Bertin · X. Draye
Earth and Life Institute, Université catholique de Louvain, Croix du Sud L7.05.11, 1348 Louvain-la-Neuve, Belgium

O. Maudoux · C. Notté
Chicoline-Cosucra, Rue de la Sucrerie 1, 7740 Warcoing, Belgium

Introduction

Fructans are the second most abundant reserve carbohydrate among higher plants. They are linear or branched fructan polymers (Vijn and Smeekens 1999) used as an alternative to starch by approximately 15 % of flowering plants, but also by some bacteria and fungi (Hendry 1993). Inulin, the main commercial fructan polymer, consists of a linear chain of beta 2-1 linked fructosyl units with a single terminal glucose. Inulin is used by the food industry as fat replacer (Arcia et al. 2011; Nowak et al. 2007) or low calories sweetener depending on the length of inulin molecules, while the pharmaceutical industry exploits its health promoting properties (Clark et al. 2012; Closa-Monasterolo et al. 2013; Di Bartolomeo et al. 2013).

Average inulin polymerization degree (DP) varies between species, but also according to developmental stage and environmental conditions. In vivo, average inulin polymerization degree (M_{DP}) in *Cynara scolymus* is 8–10 while M_{DP} is around 65 in *Helianthus tuberosus* (Hellwege et al. 1998). In chicory, M_{DP} ranges between 10 and 20 (Flamm et al. 2001) and is highly influenced by harvest date. Recently, Van Arkel described three phases in inulin

metabolism: (1) the onset of inulin biosynthesis one month after sowing with accumulation of high DP inulin until mid-September; (2) from mid-September to early November, gradual decrease of average inulin DP resulting from the synthesis of shorter inulin molecules by back-transfer activity of fructan:fructan 1-fructosyltransferase enzyme (1-FFT) (2.4.1.100) and a gradual increase in sucrose concentration; (3) inulin breakdown following exposure to cold and sub-zero temperatures associated with onset of fructan exohydrolase activity (FEH), coupled to increase in fructose and sucrose concentrations (van Arkel et al. 2012). Similar findings relative to the variation of inulin chain length during growing season but also post-harvest were reported for *Helianthus tuberosus* (Clausen et al. 2012; Saengthongpinit and Sajjaanantakul 2005) and *Cynara scolymus* (Leroy et al. 2010). Post-harvest fructan depolymerization might be considered as positive in *C. scolymus* because of the reduction of intestinal undesired side effects related to consumption of long fructan chains (Leroy et al. 2010) while this is clearly a problem in industrial chicory (*Cichorium intybus*) which is the main commercial source of inulin. In chicory, Van den Ende reports that in autumn, fructan degrading enzymes (FEH) are activated which increases free fructose and reduces average inulin chain length. High fructose concentration also results in the generation of reducing glucose-free inulo-n-ose series which can participate in the Maillard reaction with amino acids during processing. As a result, for large-scale inulin production, early harvest and a quick processing are necessary (Van den Ende and Van Laere 2002). Autumn corresponds to a change in day-length and exposure to cold and sub-zero temperatures. Leaf spoilage by cold is comparable to artificial defoliation performed at harvest, both resulting in a decrease of assimilates from source (leafs) to sink organs (taproot). These parameters were investigated in the context of end-season, post-harvest and storage to understand activation of inulin degrading enzymes. Cold temperatures and frost seem to be responsible for the activation of inulin degrading enzymes, independently of day-length, defoliation (Van den Ende and Van Laere 2002) or water stress (Vandoorne et al. 2012) as confirmed in other species (Leroy et al. 2010; Portes et al. 2008; Saengthongpinit and Sajjaanantakul 2005). In chicory, Northern blot studies showed that depolymerization is correlated to an increase of 1-FEH I (3.2.1.153) and 1-FEH II (3.2.1.153 and 3.2.1.80) transcripts (Michiels et al. 2004; Van den Ende et al. 2000; Van Laere and Van den Ende 2002) as well as to a decreased transcription of sucrose:sucrose 1-fructosyl transferase (1-SST) (2.4.1.99), the enzyme responsible for the initiation step of fructan biosynthesis. These results were correlated with enzymatic activities, evidencing a transcriptional regulation of inulin metabolism enzymes in chicory (Van den Ende and Van Laere 2002). However, 1-FEH IIa is expected to be more specifically regulated by cold (Van Laere and Van den Ende 2002).

In addition to harvest date, inulin size reduction in chicory following exposure to cold temperature is significantly cultivar-dependent (Baert 1997). In the last years, advances have been achieved in the understanding of the sequence-activity relationship of the members of the family 32 glycoside hydrolases (Van den Ende et al. 2009a; Verhaest et al. 2005), but they do not explain the genetic basis of inter-varietal variability of inulin content and properties commonly exemplified in the official varietal field tests reports (SPW-Portail de l'Agriculture wallonne 2013; van den Brink and Wilting 2012; Vlanderen-Landbouw en Visserij 2013). The first attempt to tackle this question was published recently and concerns the expression levels of 1-FEH IIa/b group which appears to be variable between individuals and tissues of a same chicory cultivar (Maroufi et al. 2010) as well as between cultivars (Maroufi et al. 2012). However, in this last paper, correlation of FEH q-RT PCR data with post-harvest carbohydrates contents and properties was not investigated nor the possible role of other GH32.

In an effort to identify the genetic bases of inter-individual variability on the susceptibility to post-harvest inulin depolymerization, we first looked for nucleotidic polymorphisms within nine fructosyltransferases from the GH32 family: they namely includes sucrose degrading genes (invertases), inulin synthesis (1-SST and 1-FFT) and inulin degrading genes (1-FEH). We then tried to identify any statistical association between the nucleotidic polymorphisms and free sugars and inulin content and properties taking advantage of two phenotypically contrasted breeding lines and later on, for confirmation purpose, of a large collection of 116 unrelated industrial chicory lines. Our results support the implication of the FEH multigenic family.

Materials and methods

Sample sets

Two sample sets were used during this study.

The "first sample set" was used to investigate the nucleotidic polymorphism present within the GH32 by Eco-TILLING. This set consists of two groups of respectively eight and twelve breeding lines (Chicoline®) selected for their contrasting susceptibility to post-harvest depolymerization of inulin. One was considered as depolymerization-susceptible (DS) while the other was considered as resistant to post-harvest inulin depolymerization (DR). The 20 breeding lines were sown on May 4th, 2007 in Pecq (7740, Belgium) and harvested on November 12th, 2007. Twelve plants per line were harvested following exposure to low

temperatures. Ten roots per line were pooled and mixed together for carbohydrates characterization (see Dedicated section). The two remaining individuals were sampled for DNA extraction and further EcoTILLING analyses.

A second sample set was used to further investigate the association of the polymorphisms detected within the FEHs with the susceptibility to post-harvest inulin depolymerization. These lines were created after self-pollination of 116 unrelated individuals selected out of a collection of 600 individuals originating from 18 ancient chicory varieties kept in collection by Chicoline®. Selection was realized based on 15 SSR using the MStrat algorithm (Gouesnard et al. 2001). This sampling algorithm is based on molecular markers to maximize the allelic richness and genetic diversity of a group of sampled individuals. Therefore, this strategy minimizes the structure and maximizes the genetic diversity of the selected individuals. Leaf samples of the 116 parents were collected for genomic DNA extraction for the 1-FEH IIa genotyping. The 116 lines were sown on May 2nd, 2011 in Warcoing (7740, Belgium) and 6–24 roots per line were harvested between the 2nd and the 9th October, 2011.

Plant Material and DNA extraction

Genomic DNA was extracted from chicory root or leaf powder (manual grinding in liquid nitrogen) according to a typical CTAB 2X procedure (Murray and Thompson 1980) with two additional PCI extractions (Phenol 25:Chloroform 24:Isoamylic alcohol 1).

Carbohydrate characterization

For all the samples used in this study, polysaccharides analyses were performed according to Van Waes (Van Waes et al. 1998). For each breeding line, carbohydrate characterization was performed at harvest and after a cold exposure.

The data were used to determine the following parameters where TF stands for total fructose (after hydrolysis), TG-total glucose (after hydrolysis), FF-free fructose, FG-free glucose and Suc for sucrose (expressed in percentage of fresh weight):

$$\text{Inulin content(fresh weight) INc} = ((\text{TF} + \text{TG} - \text{FF} - \text{FG} - \text{Suc})/1.11) \times (\% \text{ of fresh weight})$$

$$\text{Carbohydrate content CC} = \text{INc} + \text{FF} + \text{FG} + \text{Suc (\% of fresh weight)}$$

$$\text{Polymerization degree DPin} = \big((\text{TF} - \text{FF} - 0.5265\ \text{Suc})/(\text{TG} - \text{FG} - 0.5265\ \text{Suc})\big) + 1$$

$$\text{Inulin content IN} = \text{INc/CC (\% of Carbohydrate)}$$

$$\text{Free fructose content FFn} = \text{FF/CC (\% of Carbohydrate)}$$

$$\text{Free glucose content FGn} = \text{FG/CC (\% of Carbohydrate)}$$

$$\text{Sucrose content SUCn} = \text{Suc/CC (\% of Carbohydrate).}$$

Dry matter contents were determined starting from grated chicory roots. Each sample was analyzed in duplicate. Chicory root samples were grated and homogenized. 40 g of grated chicory root were added into pre-weighted evaporating dish and left at 102 °C for 24 h. After 24 h, samples were cooled down for 2 min at room temperature and weighed (evaporating dish and dry material). Samples were set back to the drying cabinet for another 48 h and weight was compared to the 24 h data point to ensure full dehydration.

Primer design and PCR amplification

PCR primers were designed manually from cDNA sequences. Thermodynamic parameters were checked with VNTI software (Invitrogen). Primer pairs were tested on genomic DNA. Optimal annealing temperatures for all amplicons were determined with a gradient thermal cycler against cloned reference sequences to avoid background or cross amplification between targets. Table 1 lists the primer pairs used for each target region as well as the amplification size on genomic DNA. For automated detection of EcoTILLING digestion products, a single primer of each primer pair was labeled with Wellred D2, D3 or D4 infrared dyes (5′ end). PCRs were performed on chicory genomic DNA in 20 µl reaction volumes in 96 wells plates (Biorad iCycler thermocycler) with 50 ng of gDNA, 4 ppm of each primer, 0.6 U of GoTaq DNA Polymerase (Promega) using the provided colorless 5× GoTaq buffer and extension time of 45 s. Annealing temperatures and number of cycles are also detailed in Table 1.

EcoTILLING

EcoTILLING aims to analyze the natural polymorphism present inside genes or genomic regions (Comai et al. 2004). EcoTILLING was performed on selected functional candidate genes: 1-FEH I (AJ242538), 1-FEH IIa (AY323935) and 1-FEH IIb (AJ295034), three fructan exohydrolases, 1-SST (U81520) and 1-FFT (U84398), the two enzymes respectively involved in the initiation and elongation steps of inulin synthesis, Putinv (Y11124), a chicory putative invertase and finally CiFr03 (JQ082515), CiFr06 (JQ082517) and CiFr07 (JQ082518), three chicory GH32 that we recently identified and are believed to be invertases.

Table 1 List of primers and cycling conditions used for EcoTILLING of nine partial sequences of chicory GH32 genes

Target	Accession number	Size gDNA (bp)	Primer sequence	Ann. T° (°C)	Cycles
1-FEH I	AJ242538	652	F-GTCCACTCTGGGGCAACATTTC	68.0	40
			R-D4-TAACCATGAAGTCGTTAGTAACCACAG		
1-FEH IIa	AY323935	472	F-GTTATGGTGCATTTGTTGACATAGATCC	59.8	40
			R-D4-ACTTTACTTTATAACAAGAATACTCTAC		
1-FEH IIb	AJ295034	591	F-GTTATGGTGCATTTGTTGACATAGATCC	59.8	40
			R-D4-GTTACAATGGCATCATGGCATTCATG		
1-SST	U81520	606	F-TACGACATCGAAGGGGTCAT	55.0	35
			R-D2-GACGCATAGAACTTTCCGTAG		
1-FFT	U84398	835	F-CTTATGGTCCCGATATGAAGCA	55.0	35
			R-D4-ATTCCGAGAGTGTTCCATCAG		
Putinv	Y11124	665	F-GGCCCACTCTGGAATTTACGAATG	61.1	40
			R-D3-CTAAGCGTTAATTCGTCTTCAGGAAC		
CiFr03	JQ082515	629	F-ATCCAAACTCGGTCTCGGCCATTC	64.0	35
			R-D3-GGGTTAATCTCTTCCGGTAGGTCA		
CiFr06	JQ082517	464	F-D3-TCCGTGCTTGGTTGTTTTAG	60.0	36
			R-GCAATCAATCAGGAGCAATG		
CiFr07	JQ082518	655	F-CACATGGGGACATGCAATATCAACG	61.1	40
			R-D3-AAGTCGTAACCCAATTCCCACATC		

F and R refer to Forward and Reverse primers and D2, D3 and D4 are the references of the Wellred dyes used for primer labeling before analysis on Beckman Ceq8000 capillary fragment analyzer

For EcoTILLING, we designed an enzyme-based SNP detection assay using a Single Strand Specific nuclease (SSS nuclease). SSS nucleases are sequence-nonspecific enzymes that recognize mispairings in a chimeric DNA molecule obtained after denaturation and annealing of two allelic PCR products. Here, EcoTILLING was performed in two steps. The first step aimed at discriminating homo- from heterozygous samples. The amplification of the target gene was directly followed by denaturation and re-annealing. The labeled amplification products were partially digested with a single strand specific nuclease (Endo-1, Serial Genetics). Labeled digestion fragments were then separated and analyzed on a CEQ8000 capillary sequencer (Beckman Coulter) using MapMarker 1000 as internal size standard (Bioventure, MM-D1 50–1,000 bp). In a second step, samples were re-analyzed against a reference PCR product (from a cloned PCR fragment or from an individual homozygous for the analyzed region). Results of the two tests were then merged to generate a deduced co-dominant genotype.

DNA sequencing

Sequencings of FEH alleles were performed on cloned PCR products and outsourced to Macrogen Inc. according to sample submission protocol (Macrogen, The Netherlands).

Genotyping

According to statistical results obtained on the first sample set and subsequent sequencing data, a genotyping primer pair was designed to amplify a 47 bp indel located in the 3′UTR of 1-FEH IIa. This tool was used to genotype 116 unrelated chicory breeding lines to confirm the statistical association between SNPs in FEH sequences and the carbohydrates contents and properties after cold exposure. The target indel was amplified with primers F-GTT-ATG-GTG-CAT-TTG-TTG-ACA-TAG-ATC-C and R-ACT-TTA-CTT-TAT-AAC-AAG-AAT-ACT-CTA-C for 40 cycles at 59.8 °C on a Bio-Rad iCycler thermal cycler. Denaturation, annealing and extension were performed for 30 s. Duration of initial denaturation was 5 min at 95 °C and final extension was 7 min at 72 °C. PCR reactions were performed in 20 µl volumes and were typically composed of 0.6 units of GoTaq polymerase (Promega), 4 µl 5× GoTaq Buffer (Promega), 4 pm of each primer, 20 pm of each dUTP and 50 ng of gDNA. Amplicons were 6-FAM labeled and were resolved on ABi 3130XL sequencer (Applied Biosystems). Outputs were scored by eye.

Data analysis

The differences of the different carbohydrates contents and properties (inulin and free sugars) of the two susceptibility

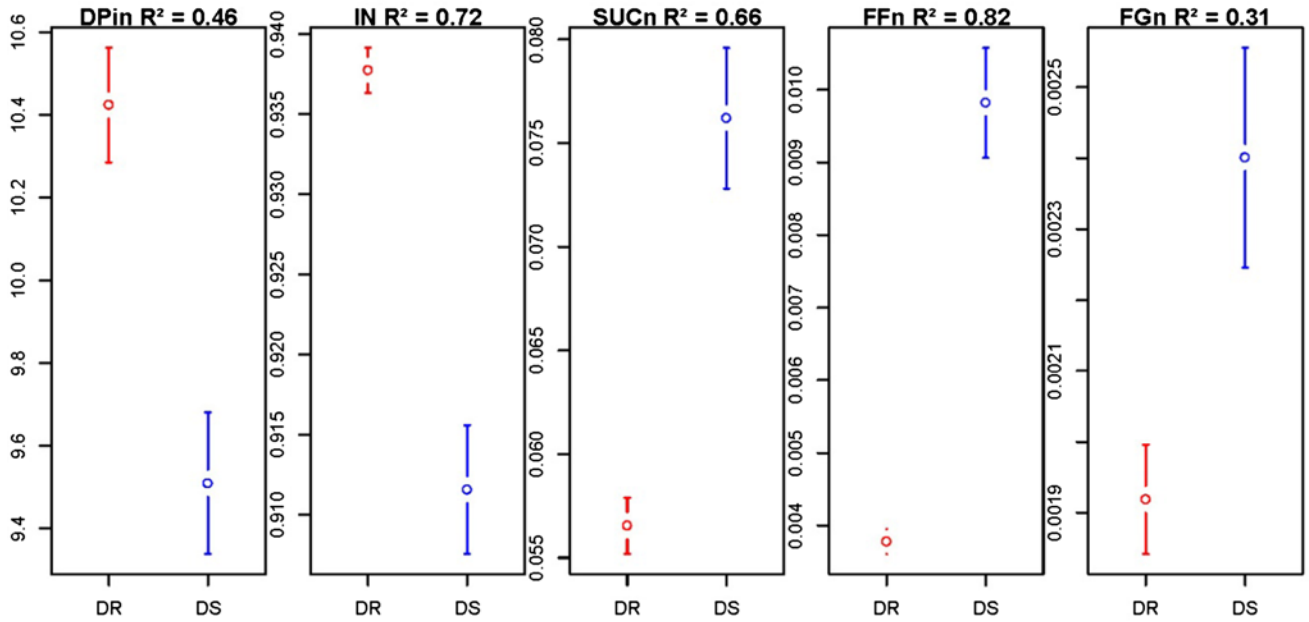

Fig. 1 Means and standard error of eight depolymerization-susceptible lines and the twelve depolymerization-resistant lines (DS vs DR, first sample set) for the post-harvest degree of polymerization of inulin (DPin), the inulin content (IN), the sucrose content (SUCn), the free fructose content (FFn) and the free glucose content (FGn). All phenotypic parameters are significantly different between the two susceptibility groups ($P < 0.006$)

groups (first sample set) were analyzed by ANOVA, using the aov function of R version 3.0.0. (R Development Core Team 2013) The potential association between the SNP loci identified by EcoTILLING and the differences in carbohydrates contents and properties were indirectly evaluated by comparing the allelic frequencies of the SNP of the two susceptibility groups with a Chi square test, using the chisq. test function of R version 3.0.0. (R Development Core Team 2013).

The association between the carbohydrates characteristics and the genotyping results obtained on the second sample set with the 1-FEH IIa marker was analyzed by ANOVA, using the aov function of R version 3.0.0. (R Development Core Team 2013). Multiple comparisons by means of Tukey were realized using the function HSD test from the agricolae package of R (De Mendiburu 2010).

Results

Polymorphism and association study

Twenty industrial chicory breeding lines exhibiting contrasted susceptibility to post-harvest inulin depolymerization were used for EcoTILLING of nine members of the GH32 multigenic family. Eight lines were considered as depolymerization-susceptible (DS) and twelve were considered as resistant to cold-induced inulin depolymerization (DR). These two subsets of breeding lines will be referred to as two "susceptibility groups". A characterization of the carbohydrate contents and properties was first performed on these two phenotypic groups to ensure phenotypic variability between the two groups. Results confirmed that, compared to the DR lines, the DS lines presented, post-harvest, significantly different carbohydrate contents with P values all lower than 0.006. The classification of the 20 breeding lines in two susceptibility groups (DS and DR) explained a high proportion of the variance of the different parameters (DPin, IN, SUCn, FGn and FFn) with R^2 ranging from 0.31 to 0.82 (Fig. 1).

EcoTILLING identified 22 SNPs and 4 insertions/deletions (Table 2). These results were compared to carbohydrate-related phenotypic groups (DS vs DR lines) to look for statistical association (Table 3). The loci 1-FEH I 76, 97, 153, 1-FEH IIa 142, 190, 301, 1-FEH IIa amplification product (430/473) and 1-FEH IIb 94 showed a clear cut segregation of the SNP alleles between the DS and DR breeding lines: SNP alleles were strictly identical within all susceptible line (DS), but were different from those of the resistant lines (DR) with χ^2 P value of 3×10^{-18}. The two last polymorphic loci located within the 1-FEH IIb gene (178, 234) were also significantly correlated, but with slightly different P values for the χ^2 test ranging from 1.3×10^{-9} to 5.2×10^{-10}. None of the others polymorphic loci analyzed by EcoTILLING showed any significant correlation with the two susceptibility groups (DS/

Table 2 Results of EcoTILLING analyzes on 9 chicory GH32s performed on 20 chicory breeding lines exhibiting contrasting susceptibility to post-harvest inulin depolymerization

		AJ242538					AY323935					AJ295034				U81520		U84398		Y11124					JQ082515			JQ082517				JQ082518	
	DS/DR	1-FEH I				AP	1-FEH IIa				AP	1-FEH IIb			AP	1-SST	AP	1-FFT	AP	Putiny				AP	CiFr03		AP	Cifr06			AP	CiFr07	AP
Reference		76	97	153	472	653	142	190	301	430/473		94	178	234	585	267	605	178	826/834	100	102	320	585	669	223	406	600/630	136	204	325	457/460	279	657
08FEH-L5-07-078-A	DS	BB	BB	BB	BB	653	BB	BB	BB	473	BB		AB	AB	585	AB	605	AB	826/834	AB	AB	AB	AB	669	AB	AB	630	AA	AB	AA	460/460	AB	657
08FEH-L5-07-078-B	DS	BB	BB	BB	BB	653	BB	BB	BB	473	BB		AB	AB	585	AA	605	AA	826/826	AA	AA	AA	AA	669	AA	AA	630	AA	AA	AA	460/460	BB	657
08FEH-L6-07-079-A	DS	BB	BB	BB	BB	653	BB	BB	BB	473	BB		AB	AB	585	AB	605	AB	826/834	AA	AA	AB	AB	669	AB	AA	630	AB	AB	AB	457/460	AB	657
08FEH-L6-07-079-B	DS	BB	BB	BB	BB	653	BB	BB	BB	473	BB		AB	AB	585	BB	605	BB	834/834	AA	AA	AB	AB	669	AB	AA	630	AA	AA	AA	460/460	AA	657
08FEH-L7-07-080-A	DS	BB	BB	BB	BB	653	BB	BB	BB	473	BB		AA	AA	585	BB	605	BB	834/834	AA	AA	AB	AB	669	AB	AA	630	AA	AA	AA	460/460	AA	657
08FEH-L7-07-080-B	DS	BB	BB	BB	BB	653	BB	BB	BB	473	BB		AA	AA	585	AB	605	AB	826/834	AA	AA	AB	AB	669	AB	AA	630	AA	AA	AA	460/460	AA	657
08FEH-L8-07-081-A	DS	BB	BB	BB	BB	653	BB	BB	BB	473	BB		AA	AA	585	BB	605	BB	834/834	AA	AA	AA	AA	669	AA	AA	630	AA	AA	AA	460/460	AA	657
08FEH-L8-07-081-B	DS	BB	BB	BB	BB	653	BB	BB	BB	473	BB		AB	AB	585	AB	605	AB	826/834	AA	AA	AA	AA	669	AA	AA	630	AA	AA	AA	460/460	AA	657
08FEH-L9-07-082-A	DS	BB	BB	BB	BB	653	BB	BB	BB	473	BB		AB	AB	585	AB	605	AB	826/834	AA	AA	AB	AB	669	AB	AA	630	AB	AB	AB	457/460	AB	657
08FEH-L9-07-082-B	DS	BB	BB	BB	BB	653	BB	BB	BB	473	BB		AB	AB	585	BB	605	BB	834/834	AB	AB	AB	AB	669	AB	AB	630	AA	AB	AA	460/460	AA	657
08FEH-L10-07-083-A	DS	BB	BB	BB	BB	653	BB	BB	BB	473	BB		AA	AA	585	BB	605	BB	834/834	AB	AB	AB	AB	669	AB	AB	630	AA	AB	AA	460/460	AB	657
08FEH-L10-07-083-B	DS	BB	BB	BB	BB	653	BB	BB	BB	473	BB		AA	AA	585	BB	605	BB	834/834	AA	AA	AB	AB	669	AB	AA	630	AA	AA	AA	460/460	AB	657
08FEH-L11-07-084-A	DS	BB	BB	BB	BB	653	BB	BB	BB	473	BB		AA	AA	585	BB	605	BB	834/834	AB	AB	BB	BB	669	BB	AB	630	AB	AB	AA	460/460	AB	657
08FEH-L11-07-084-B	DS	BB	BB	BB	BB	653	BB	BB	BB	473	BB		AB	AB	585	AB	605	AB	826/834	AA	AA	AA	AA	669	AA	AA	630	AA	AA	AA	460/460	AA	657
08FEH-L12-07-085-A	DS	BB	BB	BB	BB	653	BB	BB	BB	473	BB		AB	AB	585	AA	605	AA	826/826	AA	AA	AA	AA	669	AA	AA	630	AA	AA	AA	460/460	BB	657
08FEH-L12-07-085-B	DS	BB	BB	BB	BB	653	BB	BB	BB	473	BB		AB	AB	585	BB	605	BB	834/834	AA	AA	AB	AB	669	AB	AA	630	AA	AA	AA	460/460	AA	657
08FEH-L13-07-086-A	DR	AA	AA	AA	AA	653	AA	AA	AA	430	AA		AA	AA	585	AB	605	AB	826/834	AA	AA	AB	AB	669	AB	AA	630	AA	AA	AA	460/460	AB	657
08FEH-L13-07-086-B	DR	AA	AA	AA	AA	653	AA	AA	AA	430	AA		AA	AA	585	AB	605	AB	826/834	AA	AA	AB	AB	669	AB	AA	630	AA	AA	AA	460/460	AB	657
08FEH-L14-07-087-A	DR	AA	AA	AA	AB	653	AA	AA	AA	430	AA		AA	AA	585	AB	605	AB	826/834	AA	AA	AB	AB	669	AB	AA	630	AB	AB	AB	457/460	BB	657
08FEH-L14-07-087-B	DR	AA	AA	AA	AB	653	AA	AA	AA	430	AA		AA	AA	585	AA	605	AA	826/826	AA	AA	AA	AA	669	AA	AA	600/630	AA	AA	AA	460/460	BB	657
08FEH-L15-07-088-A	DR	AA	AA	AA	AB	653	AA	AA	AA	430	AA		AA	AA	585	AB	605	AB	826/834	AA	AA	AB	AB	669	AB	AA	630	AA	AA	AA	460/460	AB	657
08FEH-L15-07-088-B	DR	AA	AA	AA	AB	653	AA	AA	AA	430	AA		AA	AA	585	AB	605	AB	826/834	AA	AA	AA	AA	669	AA	AA	630	AA	AA	AA	460/460	AB	657
08FEH-L16-07-089-A	DR	AA	AA	AA	AB	653	AA	AA	AA	430	AA		AA	AA	585	AB	605	AB	826/834	AB	AB	BB	BB	669	BB	AB	630	AB	AB	AA	460/460	AB	657
08FEH-L16-07-089-B	DR	AA	AA	AA	AB	653	AA	AA	AA	430	AA		AA	AA	585	AB	605	AB	826/834	BB	BB	BB	BB	669	BB	BB	630	AB	AB	AA	460/460	AB	657
08FEH-L17-07-090-A	DR	AA	AA	AA	AB	653	AA	AA	AA	430	AA		AA	AA	585	AB	605	AB	826/834	AA	AA	AB	AB	669	BB	AB	630	AB	AB	AB	457/460	AA	657
08FEH-L17-07-090-B	DR	AA	AA	AA	AA	653	AA	AA	AA	430	AA		AA	AA	585	AB	605	AB	826/834	AB	AB	BB	BB	669	AB	AB	630	AB	AB	AB	457/460	BB	657
08FEH-L18-07-091-A	DR	AA	AA	AA	AB	653	AA	AA	AA	430	AA		AA	AA	585	BB	605	BB	834/834	AA	AA	AA	AA	669	AA	AA	600/630	AA	AA	AA	460/460	AB	657
08FEH-L18-07-091-B	DR	AA	AA	AA	AB	653	AA	AA	AA	430	AA		AA	AA	585	AB	605	AB	826/834	AA	AA	AA	AA	669	AA	AA	600/630	AA	AA	AA	460/460	BB	657
08FEH-L19-07-092-A	DR	AA	AA	AA	AB	653	AA	AA	AA	430	AA		AA	AA	585	AB	605	AB	826/834	AA	AA	AA	AA	669	AA	AA	630	AA	AA	AA	460/460	AB	657
08FEH-L19-07-092-B	DR	AA	AA	AA	AA	653	AA	AA	AA	430	AA		AA	AA	585	AA	605	AA	826/826	AA	AA	AB	AB	669	AB	AA	630	AA	AA	AA	460/460	AB	657
08FEH-L20-07-093-A	DR	AA	AA	AA	AB	653	AA	AA	AA	430	AA		AA	AA	585	AB	605	AB	826/834	AA	AA	AB	AB	669	BB	AB	630	AB	AB	AB	457/460	AA	657
08FEH-L20-07-093-B	DR	AA	AA	AA	AA	653	AA	AA	AA	430	AA		AA	AA	585	AB	605	AB	826/834	BB	BB	BB	BB	669	BB	BB	630	AB	AB	AA	460/460	AB	657
08FEH-L21-07-094-A	DR	AA	AA	AA	AA	653	AA	AA	AA	430	AA		AA	AA	585	AA	605	AA	826/826	AA	AA	AB	AB	669	AB	AA	630	AA	AA	AA	460/460	BB	657
08FEH-L21-07-094-B	DR	AA	AA	AA	AA	653	AA	AA	AA	430	AA		AA	AA	585	AA	605	AA	826/826	AA	AA	AA	AA	669	AA	AA	630	AA	AA	AA	460/460	BB	657
08FEH-L22-07-095-A	DR	AA	AA	AA	AA	653	AA	AA	AA	430	AA		AA	AA	585	BB	605	BB	834/834	AB	AB	AB	AB	669	AB	AB	630	AA	AB	AA	460/460	AA	657
08FEH-L22-07-095-B	DR	AA	AA	AA	AB	653	AA	AA	AA	430	AA		AA	AA	585	AB	605	AB	826/834	AA	AA	AA	AA	669	AA	AA	630	AA	AA	AA	460/460	AB	657
08FEH-L23-07-096-A	DR	AA	AA	AA	AA	653	AA	AA	AA	430	AA		AA	AA	585	BB	605	BB	834/834	AB	AB	AB	AB	669	AB	AB	630	AA	AB	AA	460/460	AA	657
08FEH-L23-07-096-B	DR	AA	AA	AA	AA	653	AA	AA	AA	430	AA		AA	AA	585	BB	605	BB	834/834	AA	AA	AB	AB	669	AB	AA	630	AB	AB	AB	457/460	AB	657
08FEH-L24-07-097-A	DR	AA	AA	AA	AB	653	AA	AA	AA	430	AA		AA	AA	585	AA	605	AA	826/826	AA	AA	AA	AA	669	AA	AA	630	AA	AA	AA	460/460	BB	657
08FEH-L24-07-097-B	DR	AA	AA	AA	AA	653	AA	AA	AA	430	AA		AA	AA	585	AA	605	AA	826/826	AA	AA	AA	AA	669	AA	AA	600/630	AA	AA	AA	460/460	BB	657

Two individuals per breeding line were analyzed, respectively, A and B at the end of the name of the sample (first column). The samples were depolymerization-susceptible (DS) or depolymerization-resistant (DR) in the second column. Each column corresponds either to (1) a SNP position detected after scoring of the EcoTILLING digestion profiles or (2) to the estimated size of the undigested amplification products as listed in the "AP" columns. SNP results are presented as "AA" or "BB" when homozygous or "AB" when heterozygous

DR). Fructan exohydrolases are, therefore, good candidates for inter-individual variability to cold-induced inulin depolymerization.

FEH sequencing

In an attempt to identify a causative mutation among the detected SNPs, the EcoTILLING fragments of the three FEHs with statistically significant SNPs were sequenced on three samples originating from the DS and DR breeding lines. No polymorphism was detected within the susceptibility groups, but several SNPs and indels were identified between the DS and DR lines. Alignments were performed comparing chicory cDNA sequences already present at NCBI and the partial gDNA sequences of 1-FEH I, 1-FEH IIa and 1-FEH IIb obtained during this study. Sequence alignments are presented in Online Resources 1–3. SNPs were detected in both introns and exons. The published cDNA sequences of the three genes (AJ242538, AJ295033 and AJ295034) almost perfectly matched the alleles identified in DS lines, while they presented several SNP when compared to the alleles present in DR lines.

The 1-FEH I EcoTILLING fragment covers three conserved regions, namely WIN**D**PNG, FR**D**P and W**E**CPD that contain the three catalytic residues of GH32 enzymes. None of the identified polymorphisms in 1-FEH I was located in these conserved regions (Online Resource 1). However, four non-silent SNPs were located close to the WECPD sequence which could affect the environment of the E residue in the active site. Attempts to identify, by homology modeling, any putative structural modifications induced by these mutations were unsuccessful (not shown). These two partial genomic sequences are accessible at NCBI under accession numbers JQ585636 (1-FEH-I-R) and JQ585637 (1-FEH-I-S).

1-FEH IIa and 1-FEH IIb EcoTILLING fragments were located across the last two exons, including a significant part of the 3′UTR region and one short intron.

Out of the six SNP mutations identified in 1-FEH IIa, only one induced a modification of the amino-acid sequence. However, a 47 bp duplication was also observed in the 3′UTR of depolymerization-susceptible 1-FEH IIa alleles (D+) compared to depolymerization-resistant 1-FEH IIa alleles (d−) (Online Resource 2). The "long" 1-FEH IIa allele will be referred to as the "D+" allele (presence of the duplication) while the "short" 1-FEH IIa allele will be the "d−" allele (for absence of duplication). This 47 bp region is a perfect tandem repeat located in the long 1-FEH IIa allele (D+/JQ585639) while a single copy is present in the shorter allele (d−/JQ585638). This 47 bp

Table 3 Referring to the EcoTILLING results (Table 2)

Target	Locus	χ value
FEHI	76	75.9***
FEHI	97	75.9***
FEHI	153	75.9***
FEHI	472	38.6***
FEHIIa	142	75.9***
FEHIIa	190	75.9***
FEHIIa	301	75.9***
FEHIIaAP	430/473	75.9***
FEHIIa	94	75.9***
FEHIIa	178	36.7***
FEHIIa	234	36.7***
1-SST	267	3.2
1-FFT	178	3.2
FFT_AP	826/834	3.2
Putinv	100	0.0
Putinv	102	0.0
Putinv	320	0.0
Putinv	585	0.0
CiFr03	223	0.0
CiFr03	406	0.2
CiFr03	600/630	4.2
Cifr06	136	1.4
Cifr06	204	0.0
Cifr06	325	0.4
CiFr06	457/460	0.4
CiFr07	279	4.6

χ^2 values evaluating the distribution of the alleles (26 polymorphic loci) among the two susceptibility group (first sample set: DS vs DR). Statistically significant effect is indicated * $P < 0.01$. ** $P < 0.001$. *** $P < 0.0001$

sequence comprises the six last codons of the cds (including the terminal TAA stop codon) and the 29 first bp of the 3′UTR. The sequence we identified in susceptible breeding lines (JQ585639) was 100 % identical to the 3′UTR of previously published AJ295033 while the partial 3′ UTR of the alleles identified in depolymerization-resistant lines (JQ585638) contained 2 SNPs within their single copy of the 47 bp region.

Out of the 10 SNP mutations identified in 1-FEH IIb, only two were non-silent while seven were silent and a last one was an indel of 1 bp in an intron (Online Resource 3). 1-FEH IIb sequences have accession numbers JQ585640 (1-FEH-IIb-S) and JQ585641 (1-FEH-IIb-R).

Confirmation of the association

In absence of any detected causative mutation in the three FEHs examined and considering the likely physical linkage between these three genes, the ease of scoring a 47 bp indel and the previously reported implication of 1-FEH IIa/b in cold-induced inulin depolymerization (Van Laere and Van den Ende 2002), a larger set of 116 unrelated lines was studied by genotyping only the 47 bp indel present in the 3′UTR of 1-FEH IIa to confirm the preliminary detected associations.

Carbohydrate-related phenotypic parameters were then evaluated on the progeny of 116 lines, before and after post-harvest cold exposure and compared to 1-FEH IIa genotyping results.

At harvest, the average dry matter and carbohydrates content were 24.6 and 17.8 %, respectively, of total fresh weight of both groups of lines. The carbohydrates mainly consisted in inulin (IN = 94.6 %) and to a lower proportion of sucrose (SUCn = 4.9 %). The free fructose (FFn) and free glucose (FGn) represented only 0.33 and 0.17 % of the carbohydrates. The average degree of polymerization (DPin) was 12.2. At this stage, the nature of the 1-FEH IIa genotype was not significantly correlated with any of these parameters ($\alpha = 0.01$) (Online Resource 4).

The dry matter and carbohydrate contents were not significantly different after the cold exposure but we observed (1) a reduced DPin and inulin content (IN) (2) an important increase in sucrose (SUCn) and fructose (FFn) concentrations and (3) a slight increase in glucose (FGn) concentration (see Fig. 2). These variations were all significantly correlated to the nature of the 1-FEH IIa genotype (P value ranging from 4.2×10^{-6} to 4.1×10^{-12}). More precisely, the homozygous (D+/D+) had lost 25.8 % of IN and 4 U of DPin after the cold exposure, while the homozygous (d−/d−) had lost only 10.8 % of IN and 0.69 U of DPin. The sucrose (SUCn), fructose (FFn) and glucose (FGn) increased by 17.8, 7.7 and 0.36 %, respectively, for the (D+/D+) genotype, and by only 7.43, 3.22 and 0.129 % for the (d−/d−) genotype. The heterozygous (D+/d−) genotype presented an intermediate phenotype for IN, SS and SUCn but they clustered with (1) the homozygous (D+/D+) genotype for FFn and FGn and (2) the (d−/d−) genotype for DPin. Presence or absence of the 47 bp indel in 1-FEH IIa explained a high proportion of IN and SUCn variation following cold storage ($R^2_{IN} = 0.35$ and $R^2_{SUCn} = 0.36$) and a lower proportion for the DPin, FFn and FGn ($R^2_{DPin} = 0.18$, $R^2_{FFn} = 0.23$ and $R^2_{FGn} = 0.22$).

Discussion

Transcriptional regulation of inulin synthesis and degradation genes belonging to the GH32 family of hydrolases in response to late season exposure to cold temperature has been reported (Van den Ende et al. 2002). Even if these results pinpoint clear candidate genes for cold-induced

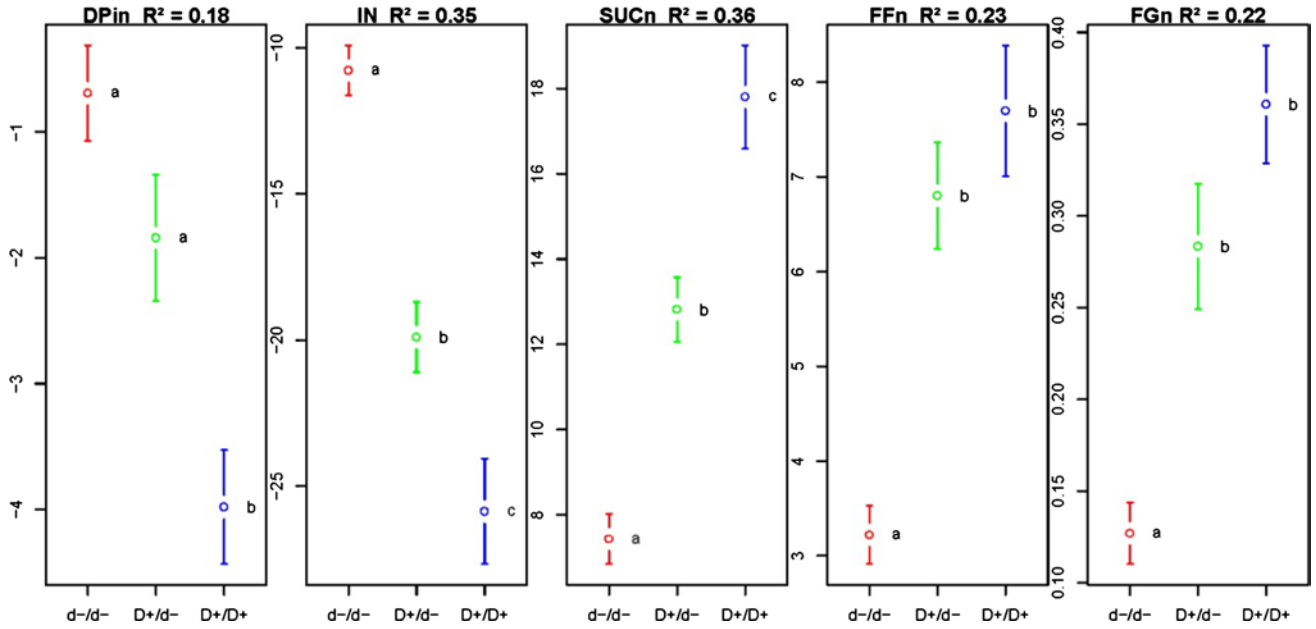

Fig. 2 Means and standard error of the three different 1-FEH IIa genotypes ("d−" refers to the allele "absence of duplication" and the "D+" to the allele "presence of the duplication") among 116 lines for the differences before and after cold exposure of the inulin degree of polymerization (DPin), inulin content (IN), sucrose content (SUCn), free fructose content (FFn) and free glucose content (FGn). The means followed by different letters were significantly different ($P < 0.01$). The 1-FEH IIa genotype significantly affected all five investigated phenotypic parameters ($P < 0.00001$)

inulin depolymerization, they do not explain inter-individual variability of susceptibility to post-harvest inulin depolymerization.

In the past, the importance of point mutations on the activity of GH32 was investigated and residues implicated in the active site, substrate specificity and regulation were described (Ritsema et al. 2006; Van den Ende and Valluru 2009; Verhaest et al. 2007). However, the relationship between GH32 point mutations and the susceptibility to post-harvest inulin was not identified by these authors.

This research was composed of two distinct, but complementary, steps. A first step consisted in the investigation of the natural polymorphism present among several representative members of the GH32 multigenic family in chicory. We then evaluated the possible association between the identified polymorphisms and post-harvest phenotypic variability of chicory root carbohydrates content and properties. This first step was conducted on a limited number (20) of phenotypically contrasted breeding lines. This first step allowed identifying statistically significant polymorphisms within three strong candidate genes, namely 1-FEH I, 1-FEH IIa and 1-FEH IIb. In the second part of this research, based on these preliminary results, a genotyping probe targeting an indel located inside 1-FEH IIa was developed and used on a collection of 116 unrelated chicory lines selected to maximize genetic diversity and minimize genetic structure to confirm the detected genotype/phenotype associations.

Inulin chain length in the root is believed to be affected by both synthesis and depolymerization mechanisms (Van den Ende et al. 1996). However, our results did not identify any statistical association between 1-SST or 1-FFT polymorphisms, the two key enzymes responsible for the initiation and elongation of inulin, and the post-harvest average inulin chain length or free sugars content.

At the level of degrading enzymes, two distinct classes were investigated, namely invertases and fructan exohydrolases. Invertases are sucrose cleaving enzymes. Their activity modifies sucrose availability which could affect synthesis process, and increase free glucose and free fructose. Fructan exohydrolases are inulin degrading enzymes. Their activity results in a general decrease of the average inulin chain length and in an increase of the free fructose and sucrose.

Based on its high sequence homology to a *Daucus carota* beta-fructofuranosidase (X75352/CAA53098), CiFr07, a chicory GH32 that we recently identified, could be an acid vacuolar invertase. The unique SNP identified in CiFr07 was not associated with a post-harvest difference of the degree of polymerization of inulin (DPin) or small sugar content (FGn, FFn, SUCn).

The last six sequences that were investigated all belong to the group of FEH/cell wall invertases. Fructan hydrolysis

genes are believed to have evolved from cell wall invertase and gained sucrose inhibition (1-FEH IIa and IIb) with specificity for β-2,1 linear fructan molecules. 1-FEH I is also a fructan hydrolyzing enzyme, but with a weaker susceptibility to sucrose inhibition. For this study, we also included two new chicory GH32, CiFr03 and CiFr06 that are likely to be cell wall invertases since they exhibit the S101 to I101 substitution in the GSAT-YTG conserved regions, which is associated with the absence of sucrose inhibition (Verhaest et al. 2007). The presence the D−-K/R motif specific to cell wall invertases also supports this assignation (Van den Ende et al. 2009b). Polymorphisms detected in CiFr03 and CiFr06 lacked statistical association to phenotypic data. Putinv (Y11124) was the sequence presenting the highest homology to CiFr03 and CiFr06 (80 %) and did not show any statistical association either.

We found strong statistical association in the first small set of genotypes between nucleotidic polymorphisms located within three FEHs (1-FEH I, 1-FEH IIa and 1-FEH IIb) and carbohydrate parameters. Partial genomic sequencing of these three FEHs identified a 47 bp indel within the 3′UTR of 1-FEH IIa. Based on in silico secondary structure predictions, one can speculate that the presence of a perfect tandem repeat in the 3′ UTR of 1-FEH IIa could generate a second hairpin structure (Online Resource 5) and possibly affect m-RNA stability or pre-mRNA processing as reported previously (Gutierrez et al. 1999; Millevoi and Vagner 2009).

We also observed an apparent linkage of the genotyping results observed between the three FEHs (Table 2) suggesting a possible physical proximity of the three genes. Previous results reported physical proximity of 1-FEH IIa and 1-FEH IIb located at 28.8 and 30.6 cM, respectively, on LG4 of the Rubis 118 physical map of chicory (Cadalen et al. 2010). In lettuce (CGPDB 2013), 1-FEH I and 1-FEH II genes are located about 40 kb from each other (CGBPD, Davis. 1-FEH I, LG5 116059887-116060805, 1-FEH II LG5 116092584-116093466), which, considering the existing synteny between the two species (Muys et al. 2013), might support the physical proximity of the three FEHs. Considering the high redundancy observed between statistically significant SNP loci located in the three FEHs, taking into account previous data supporting the role of 1-FEH II in cold-induced inulin depolymerization and the ease of scoring a large indel, we decided to focus on 1-FEH IIa.

The statistical association between 1-FEH II and post-harvest inulin depolymerization identified on the first sample set was confirmed on a second, wider, genetically diversified and unstructured collection of 116 industrial chicory lines. In this study, total carbohydrate content was identical before and after cold storage, while inulin and free sugars contents were modified. Post-harvest, the 1-FEH IIa marker explained approximately 35 % of the variance of inulin and sucrose contents. For these carbohydrates, the three genotypes (d−/d−, D+/d− and D+/D+) were statistically different, suggesting a co-dominant effect of the causative loci. This additive effect was less clear for the inulin degree of polymerization (DPin), free fructose (FFn) and free glucose (FGn) contents for which R^2 values were around 0.2.

An intriguing result of this study concerns the free carbohydrates content post-harvest. The drop in inulin content resulted in an equivalent increase of free sugars. However, free fructose and to a smaller extent sucrose (end product of inulin depolymerization by FEH enzymes) should have increased much more than observed. Fructose increase represented only 1/3 of the increase, while sucrose accounted for 2/3 of the post-harvest increase in free sugars, coupled with a very low level of free glucose (between 0.1 and 0.4 %). This raises a question about recycling of the free fructose generated during inulin hydrolysis. Free fructose might have been converted to sucrose by sucrose synthase (EC 2.4.1.13) or sucrose phosphate synthase (EC 2.4.1.14), resulting in low levels of free glucose and an increase in sucrose (Nguyen-Quoc and Foyer 2001).

Conclusions

A candidate-gene approach was successfully applied to identify phenotype/genotype association in the non-model species chicory. We identified several SNPs and indel polymorphisms located in three FEH genes and found a statistical association between a 47 bp indel located in the 3′UTR of 1-FEH IIa and an enhanced susceptibility to post-harvest inulin depolymerization. These results are of particular interest if we consider that these FEHs are, until now, the only functionally characterized β-2,1-hydrolyzing enzymes identified in chicory. Statistical analyses indicated that identified polymorphisms could explain up to two-thirds of the inulin loss and free sugars accumulation and save up to 5 DP units on the average inulin chain length post-harvest.

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Conflict of interest O. M. and C. N. are members of Cosucra-Group Warcoing S.A.

Ethical standards The authors acknowledge that the experiments described in this paper comply with the current laws of the country in which they were performed.

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