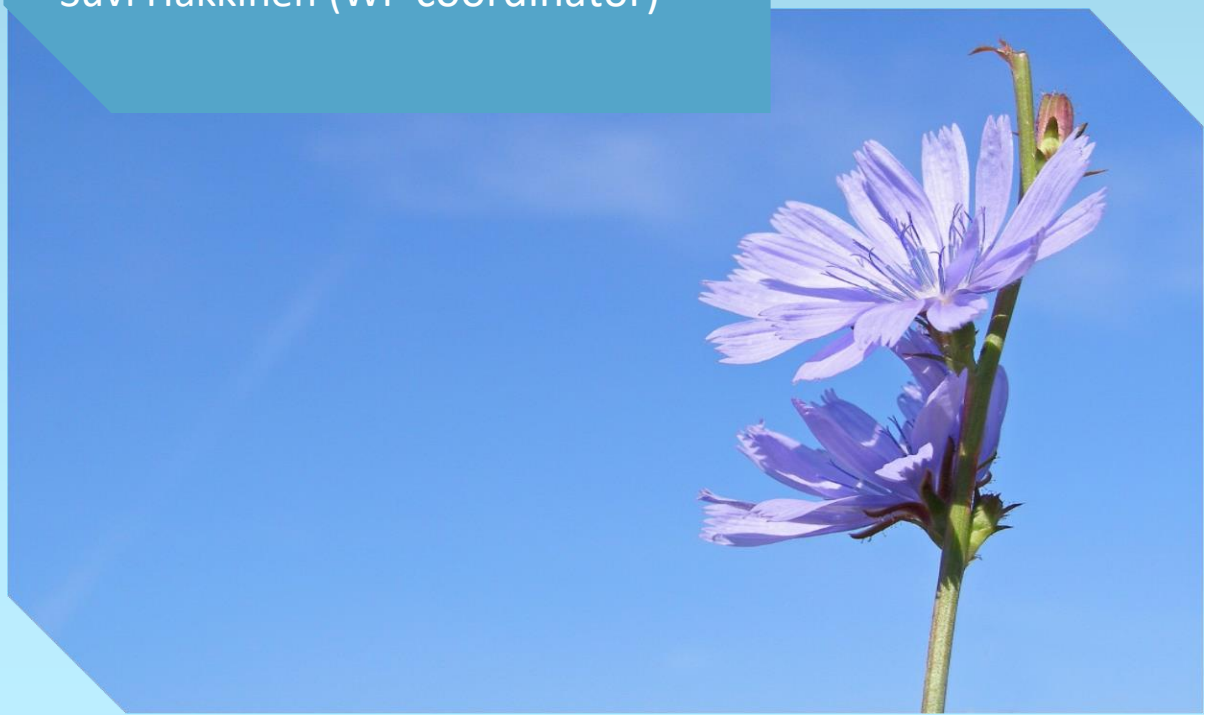
D.8.3 An overview of inulin and terpene quantity and quality in the NPBT chicory in a typical growing period comparable to the period August to December in comparison to commercial chicory varieties.

Suvi Häkkinen (WP coordinator)



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Contents

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1 Summary

This report describes terpene and inulin profiles of selected NPBT chicory varieties and their comparison to commercial chicory. Terpene profile was edited in chicory by genome editing, using the CRISPR/Cas9 system to inactivate four genes that encode the enzyme that performs the first dedicated step in sesquiterpene lactone (STL) synthesis, germacrene A synthase (*Ci*GAS). GAS-edited lines exhibited decreased amounts of STLs, while accumulation of polyphenols (PPs) was increased. Inulin biosynthesis was modified using the CRISPR-Cas9 system by knocking down the three inulin degradation genes encoding the fructan exohydrolyse enzyme 1-FEH. These genes were shown to effect inulin production under cold and stress conditions, and as the crop is harvested as late as possible in the year to have big roots, the degradation of inulin that occurs in this part of the season is very much unwanted. Both NPBT chicory varieties were compared to commercial chicory for their terpene and/or inulin contents.

2 Introduction

The major STLs of chicory belong to the class of guaianolide sesquiterpene lactones and are thought to be derived from a single sesquiterpene precursor, germacrene A (**Fig. 1**). Germacrene A does not accumulate in chicory, but is efficiently converted to the most predominant chicory STLs lactucin, lactucopicrin and 8-deoxylactucin in their oxalated forms. These STLs contribute to the bitter taste of chicory products and they need to be removed during the industrial processing of chicory roots to produce inulin, which is accounting for 30 % of the processing costs. Thus, there is a high interest in developing low-terpene chicory varieties, in order to lower the production costs of commercial inulin. In chicory, four GAS genes have been identified, one copy of GAS-long (*Ci*GAS-L) and three copies of GAS-short (*Ci*GAS-S1, *Ci*GAS-S2, *Ci*GAS-S3) (Bouwmeester et al. 2002; Bogdanović et al. 2019).

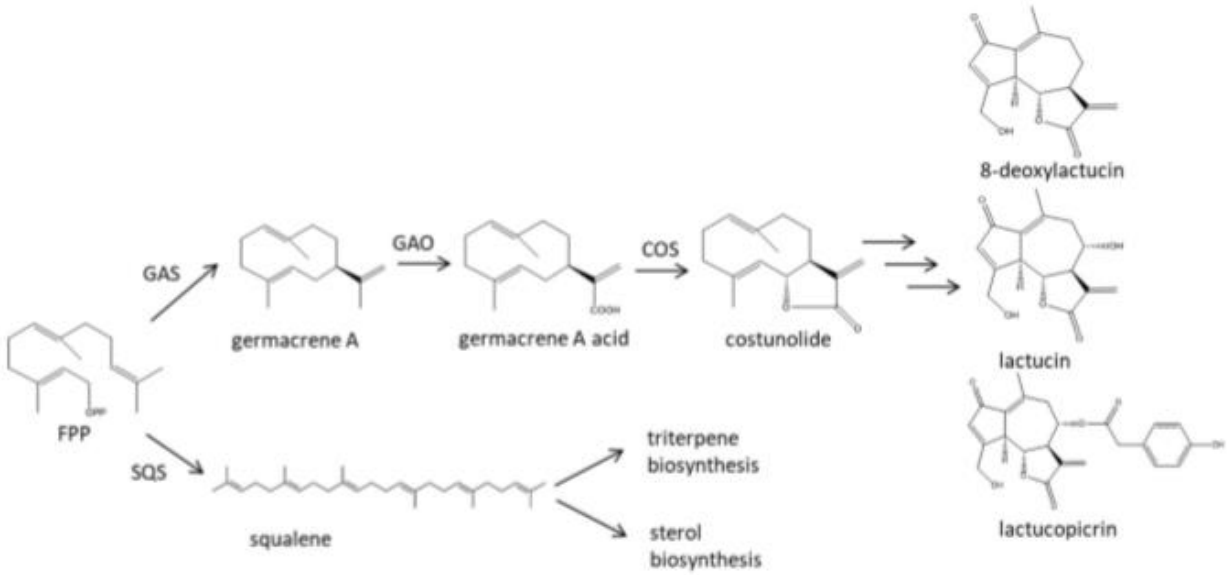

Fig. 1. Biosynthetic pathway of chicory sesquiterpene lactones. FPP – farnesyl pyrophosphate, GAS - germacrene A synthase, GAO - germacrene A oxidase, COS – costunolide synthase, SQS – squalene synthase.

Chicory is sown in spring and the taproots are harvested in autumn/ winter of the same year before the temperature drops. Upon temperature drop at the end of the growing season, inulin molecules are degraded

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by exohydrolase enzyme (1-FEH) catalysing the degradation of inulin and resulting in lower degree of polymerisation (DP) and an accumulation of small sugars, such as fructose, glucose and sucrose. In chicory, three genes are known to encode 1-FEH enzymes, 1-Feh1, 1-Feh2a and 1-Feh2b, which are differentially induced. By CRISPR/Cas9 genome editing, the 1-FEH ezyme activity was blocked, resulting in higher DP inulin, which is of higher value due to its higher stability as well as for lower undesired gut effects.

3 Materials and Methods

_Ci_GAS and FEH editing

Genome editing of *Ci*GAS was performed essentially as described in (Cankar et al. 2020). Briefly, five guide RNAs targeting different positions in exon 4 of the CiGAS-S1 gene were designed and synthesized on plasmids under control of the *A. thaliana* U6 promoter (NCBI Genbank accession numbers MH350853 - MH350858). Separate plasmid for *Sp*Cas9 expression was made, with a C- terminal NLS driven by the constitutive pParsley ubiquitin promoter. After testing for activity, three guides were selected for the mutagenesis experiments and these were synthesized together on a single plasmid separated by tRNA sequences and expressed using the *A. thaliana* U6 promoter. Protoplasts were isolated from *in vitro* shoot cultures of *Cichorium inybus* var. *sativa* (Orchies C37) and transfected with *Sp*Cas9 expression plasmid and a plasmid carrying a sgRNA cassette. After three weeks, microcalli were separated and transferred to fresh medium every 3-4 weeks until signs of regeneration appeared. The developing shootlets were harvested and rooted.

Genome editing of *Feh* genes was performed by transformation of *Cichorium intybus* var. *sativa* (Orchies C37) with a plasmid containing CRISPR-Cas9 and guide RNA sequences using *Agrobacterium*-mediated method essentially as described in van der Meer et al (1994). Briefly, four guide RNAs targeting the Feh1 and Feh2a/b genes in positions in exon 3 were used to induce indel mutations in all Feh genes. A binary vector was used with NPTII selection marker and 35Spromoter-Cas9-Nost gene construct with the four guide RNAs. Leaf material of C37 chicory was co-cultivated with *Agrobacterium* containing the CRISPR-Cas9 construct. After three weeks on selection medium the first calli developed and plant material was transferred to fresh medium every 3-4 weeks. Transgenic shoots were harvested and rooted on rooting medium and after genotyping transferred to the greenhouse. Mature mutant plants were multiplied via root cuttings and used for the FEH induction experiment.

Analyses of inulin and sugar profiles

Inulin and sugar profiles and inulin weight average polymerisation degree (DP) were determined by SEC and HPAEC-PAD-MSQ analyses using enzymatic hydrolysis as described in Willför et al. (2009). Inulin mean (mDP) was analysed by HPAEC-PAD (Dionex) in which the free glucose and fructose is analysed before and after acid or enzymatic hydrolysis of the chicory root inulin sample as described in van Arkel et al (2012). The method for determination of polymerisation degree using SEC analyses (at VTT) diverts from the determination by using glucose and fructose analysis after hydrolysis using HPAEC (performed at WR) probably because SEC analysis is known to be less sensitive to smaller inulin molecules (Evans et al, 2020 and refs. therein). Therefore, the results deriving from the former are reported as weight average DP and those from the latter are used to determine the mDP, as this method is also used by industry. Dietary fibre (DF) was analysed with ANKOM DF-equipment using Method AACC 2011.25.

Analyses of terpenes and polyphenols

Sesquiterpene lactones and phenolic compounds were analysed by LC-MS and GC-MS as described in Cankar et al. (2020 submitted), using methanol and hexane: ethyl acetate mixture (v/v 85:15), respectively.

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4 Results

Inulin profiles in *Ci*GAS-KO and FEH KO lines and WT lines

Besides the commercially available inulin, the inulin DP was determined from *Ci*GAS KO and FEH KO lines (produced in WP1 for WP2) in order to understand whether the induced modifications could affect the inulin mean DP or dietary fibre in chicory.

In *Ci*GAS KO lines the aim was to reduce terpenes while the inulin quality of these lines would stay unaltered. The weight average DP of *Ci*GAS KO lines (20-21) were indeed unaltered compared to WT lines (21). Likewise, the mDP of both WT and *Ci*GAS KO lines were equal 13-14. Dietary fibre (DF) of a WT and *Ci*GAS KO chicory line samples as such (without extraction) showed that between the three *Ci*GAS KO lines, only small differences in DF were observed and the total DF as well as soluble DF contents were high compared to literature data of other legumes (**Table 1**). This analysis confirmed that indeed the inulin profile of *Ci*GAS KO chicory lines stays unaltered although the lines do show a reduced terpene content, as expected.

***Table 1**. Dietary fibre analysis.*

	C7-150 Root		C9-4 Root		C7-12 Root		Frutafit TEX		Carrot**	Horseradish**
	Value (%)	Avedev.*	Value (%)	Avedev.*	Value (%)	Avedev.*	Value (%)	Avedev.*	Value	Value
Total dietary fibre (% dm)	84,4		81,2		73,6		98,7		19,8	26,8
Insoluble DF (% dm)	11,6	0,2	15,5	0,2	12,1	0,3	0,0		14,7	23,9
Soluble DF (% dm)	72,8		65,7		61,5		98,7		5,1	2,9
SDFP (% dm)	21,2	2,4	16,4	4,8	17,3	1,3	49,2	1,1	5,1	2,9
SDFS (% dm)	51,6		49,3		44,2		49,5		below LOQ	below LOQ
*calculated based on avedev of the residues in the first step of the analysis										
SDFP=soluble dietary fibre that precipitates (=high molecular weight soluble DF)										
SDFS=soluble dietary fibre that stays soluble (=low molecular weight soluble DF)										
** Reference value by Pastell, Putkonen and Rita (2019)										

Inulin biosynthesis was modified by knocking down all alleles for the three inulin degradation genes encoding two fructan exohydrolyse enzymes FEH1 and FEH2. These genes were shown to effect inulin biosynthesis and the subsequent inulin profile under cold and stress conditions that mimics the situation in Autumn and Winter. Since the field cultivation is currently not allowed for NPBT mutants that are seen as GM organisms, the stress conditions to mimic the Autumn and Winter conditions that normally induce the breakdown of inulin were induced in a controlled greenhouse under GM restrictions. It was observed that for WT plants, cold treatment induces Feh1 gene expression and defoliation induces Feh2. Both cold and defoliation induced all Feh genes and induced the FEH enzyme activity, thus the selected treatments were applied: Feh1 KO plants: cold (12°C/4°C, 12h day/12h night), Feh2 KO plants: defoliation, Double KO mutants: cold + defoliation. Root samples were harvested at three time-points: t=0 (N=4), t=3 weeks, no induction (N=3), and t=3 weeks, induction (N=3). For FEH KO lines, it was shown by WR (WP2) that in especially in the double KO lines, after induction a strong increase in fructose and other small sugars was observed in WT (which is undesirable), while amount in the KO lines remained low. Also, mDP in WT was decreased, while it was stable in the KO lines (**Fig. 2**). These results show that in WT inulin is depolymerised/ broken down due to the Autumn/ Winter conditions, while in double KO lines the break-down is not taking place. The profile of inulin from the roots of the double KO under the stress conditions showed a very good profile with an accent on higher DP inulin molecules and a very low concentration of free small sugars and low DP inulin, in clear contrast to control WT chicory.

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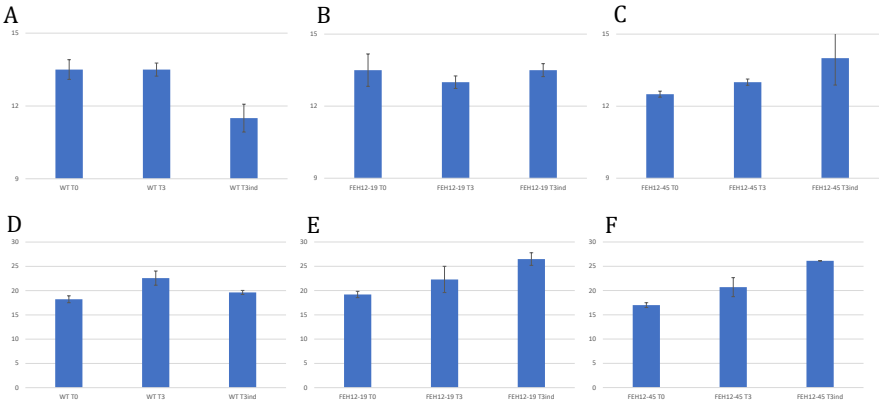

Fig. 2. FEH double KO mutants (FEH12-19 and FEH12-45) and WT at the start of the experiment (T0), after three weeks (T3) and after three weeks induction (T3ind). **A**)-**C**): inulin mDP; **D**)-**F**): inulin weight average DP.

Terpene profiles in *Ci*GAS-KO and FEH KO lines and WT lines

Sesquiterpene lactone content was determined in the roots of five *Ci*GAS mutant lines and the control plants, and it was shown that several lines containing mutations in the *Ci*GAS genes showed a strong reduction in the amount of STLs (**Fig. 3**). It is not expected to have seasonal variation seen in the terpene contents of these lines.

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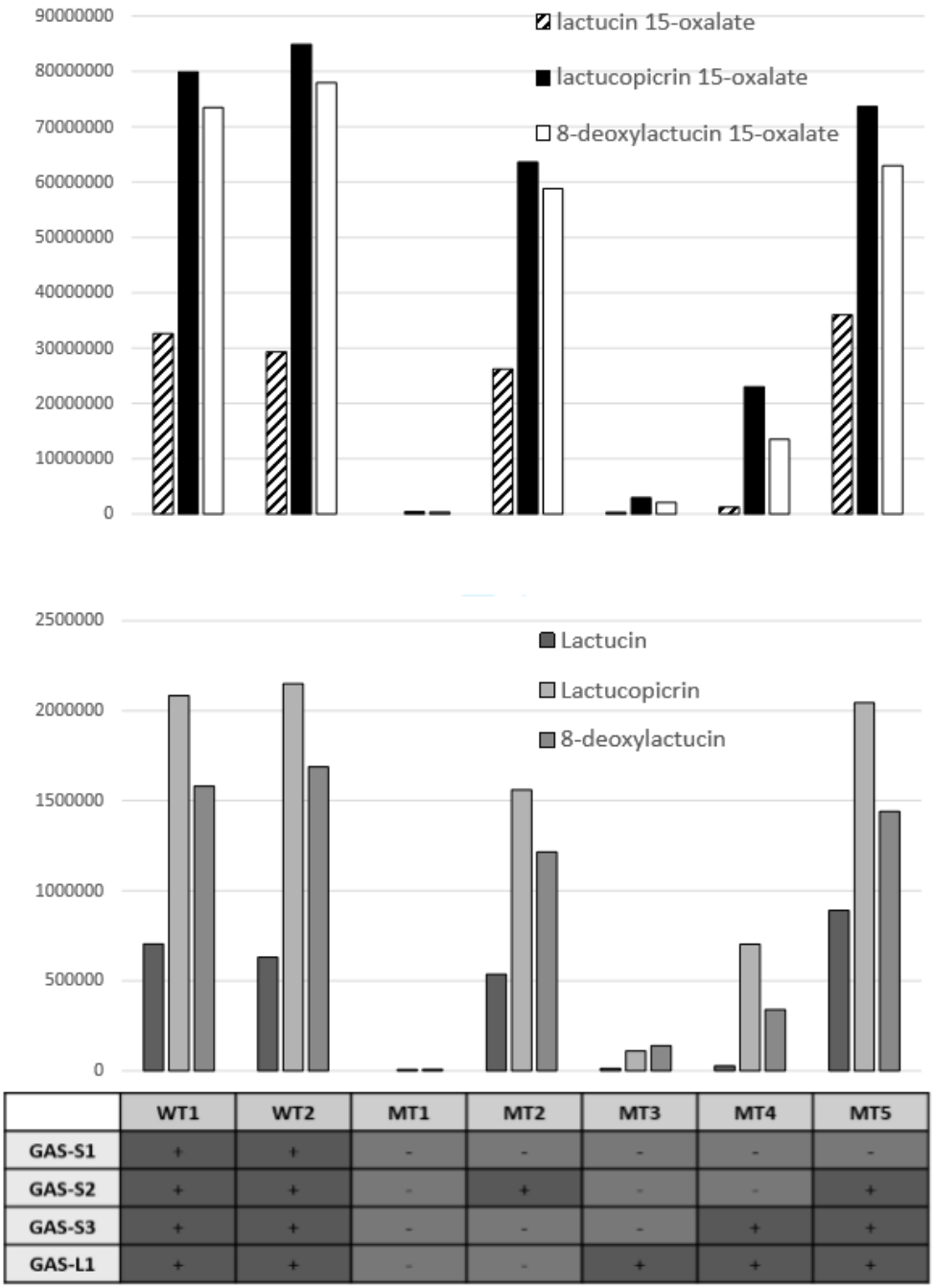

	WT1	WT2	MT1	MT2	MT3	MT4	MT5
GAS-S1	+	+	-	-	-	-	-
GAS-S2	+	+	-	+	-	-	+
GAS-S3	+	+	-	-	-	+	+
GAS-L1	+	+	-	-	+	+	+

Fig. 3. Relative amounts of STLs based on the peak area produced in *Ci*GAS and control lines (Cankar et al. 2020, submitted). Two control plants (WT1 & WT2) and five chicory lines with an indel (KO) in one or several copies of the *Ci*GAS gene (MT1 to MT5) were assayed. The genotype of each line is shown by + (gene functional, WT or heterozygous) and – (non-functional gene, homozygous mutations).

Inactivation of *Ci*GAS lead to the increased accumulation chicory polyphenols, especially in the root tissue (**Fig 4**). Chlorogenic acid accumulation increased 3.8-fold, 3.0-fold and 1.7-fold in the roots of chicory *Ci*GAS deletion

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lines MT1, MT3 and MT4, respectively (Cankar et al. 2020, submitted). 3,5-dicaffeoylquinic acid increased 5.6-fold, 4.0-fold and 1.9-fold in the roots of lines MT1, MT3 and MT4, respectively. WT levels of chlorogenic acid and 3,5dicaffeoylquinic acid were observed in roots of the lines with STL levels close to the WT levels (MT2 and MT5).

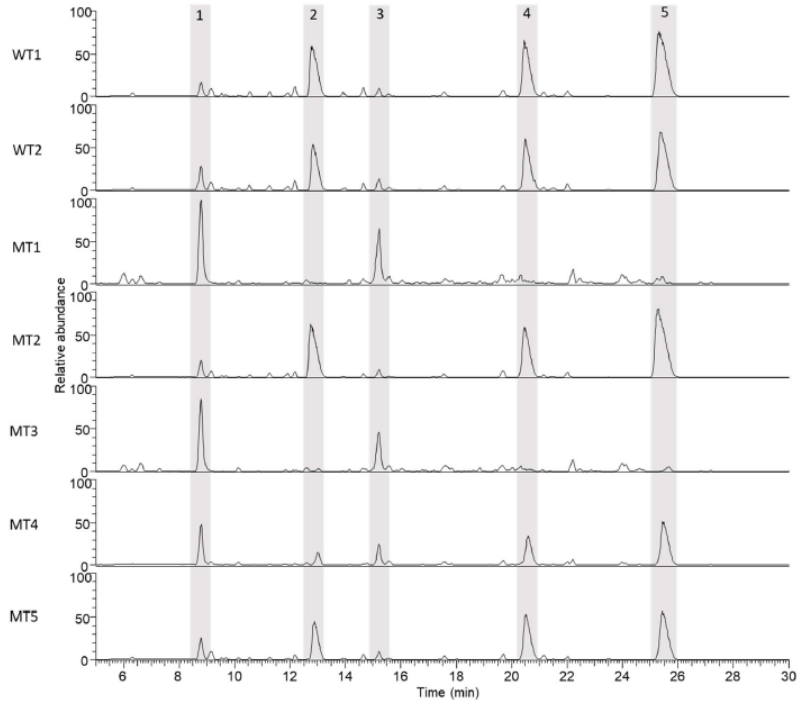

Fig. 4. LC-MS analysis of the methanolic extracts of chicory roots with *Ci*GAS deletion (Cankar et al, 2020 submitted). A full MS chromatograms of two WT lines and 5 chicory lines with a CiGAS deletion show a clear reduction of lactucin oxalate (peak 2, C17H16O8, [M+H]= 349.09179), 8-deoxylactucin-15- oxalate (peak 4, C17H16O7, [M+H]= 333.09687) and lactucopicrin-8-oxalate (peak 5, C25H22O10, [M+H]= 483.12857) in roots of chicory lines MT1, MT3 and MT4. In the same lines an increase of chlorogenic acid (Peak 1, C16H18O9 ,[M+H]= 355,10235) and 3,5-dicaffeoylquinic acid (Peak 3, C25H24O12, 517,13405) in the taproot extract is observed.

Besides alterations in STLs and PPs, GAS-edited plants exhibited higher accumulation of squalene, putatively due to the increased pool of farnesyl diphosphate (FPP) after inhibition of GAS activity (**Fig. 1**) (**Fig. 5**). Squalene is the first product in the sterol synthesis pathway, leading to the production of triterpenes and plant sterols, such as sitosterol, campesterol and stigmasterol. Plant-derived squalene is also a commercially seeked compound (Gohil et al. 2019), used in cosmetic and pharmaceutical industry, e.g. as vaccine adjuvant. Three GAS-edited lines (MT1, MT3 and MT4) accumulated squalene (55 - 154 µg/g FW), while it was not detected in WT plants (**Fig. 5**).

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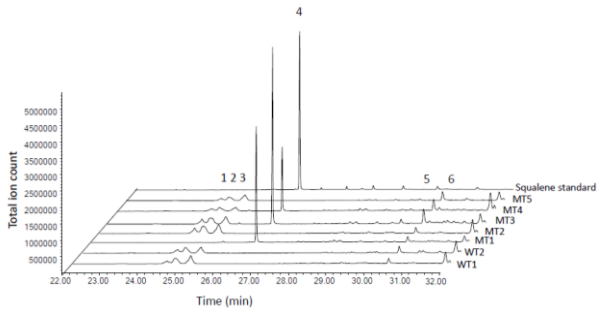

Fig. 5. GC-MS chromatogram of chicory root extracts (Cankar et al, 2020, submitted). Chromatograms of two WT and 5 CiGAS deletion lines are shown. Peak 1 – 3: acetylated triterpenes (C32H52O2, MW=468), Peak 4 – squalene, peak 5: stigmasterol, peak 6: sitosterol.

5 Conclusions

Several NPBT chicory lines, either targeting the inulin degradation (FEH KO) or for terpene biosynthesis (*Ci*GAS KO) have been established and evaluated for their inulin and terpene quality and quantity. It was shown that in *Ci*GAS KO lines strong reduction of terpenes was seen, while terpene pathway editing did not have effect on the inulin biosynthesis. In FEH KO lines, positive changes in inulin polymerisation degree and no accumulation of small sugars after subjecting roots to Autumn/Winter conditions was observed, when break down of inulin chain lenght takes place in WT chicory.

6 References

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