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Original Research

CRISPR/Cas9-Targeted Mutagenesis of *CiGAS* and *CiGAO* to Reduce Bitterness in Chicory (*Cichorium intybus* L.)

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Abstract

Background: Chicory (*Cichorium intybus* L.), a member of the Asteraceae family, is known for its numerous health benefits, including its prebiotic, digestive, antioxidant or anti-inflammatory effects. Used as a coffee substitute, chicory roots is also appreciated for its bitterness, which can prove to be a disadvantage for other uses in food. The bitterness of chicory is largely linked to the presence of sesquiterpene lactones (STLs) in the roots. **Methods**: In order to create less bitter industrial chicory varieties, CRISPR/Cas9 technology was used to inhibit the first two genes of the STL biosynthetic pathway: germacrene A synthase (*CiGAS*), short form, and germacrene A oxidase (*CiGAO*). To determine the impact of these reductions on the perception of bitterness, a sensory analysis of 13 field-grown chicories genotypes, contrasting for their STL composition, allowed the construction of obtain a bitterness scale by correlating STL content with perceived bitterness. The edited chicories were positioned on this scale according to their STL content. **Results**: Biallelic mutations in two of the copies of *CiGAS-short* form or in the *CiGAO* gene led to a reduction in STL content of edited chicories and a reduction in bitterness, or even an absence of perception, was obtained for some mutants. **Conclusions**: The use of the CRISPR/Cas9 tool as well as the choice of targets therefore makes it possible to modulate the bitterness of chicory.

Keywords: *Cichorium intybus* L.; bitterness; sesquiterpene lactones; CRISPR/Cas9; hairy roots; germacrene A synthase; germacrene A oxidase

1. Introduction

Cichorium intybus L., also known as chicory, is a species belonging to the Asteraceae family widely used in traditional medicine. Its biological activities are mainly attributed to the accumulation of inulin, vitamins, and specialized metabolites such as sesquiterpene lactones (STLs), flavonoids, coumarins, hydroxycinnamic acids as well as alkaloids present in different parts of *C. intybus* L. [1]. In Europe, different varieties of chicory are cultivated either for the consumption of their leaves or for the use of their roots. In the North of France, *C. intybus* var. *foliosum* is commonly used as a salad and the taproot of *C. intybus* var. *sativum* (or industrial chicory) is grown for industrial purposes. Roots of industrial chicory are of economic importance for roasted ingredients, beverage processing such as coffee substitute, and inulin production. The concept of functional ingredients is increasingly developing due to the attraction of consumers towards healthier food. Chicory inulin is already considered as a functional ingredient due to its health effects and recently a new trend describing chicory flour as such has been developed [2–6]. Chicory flour, produced from roots, is mainly employed as supplement in baking since it improves the taste, the preservation, and the texture of bakery products in addition to being low in calories and rich in fibers. However, its incorporation is limited to 5% because chicory bitterness becomes too perceptible in larger proportions [7]. Bitterness plays a major role in food consumption and can cause an aversive reaction in consumers. By offering less bitter varieties of chicory, it will be possible to produce less bitter ingredients from chicory and, as a consequence, to integrate more chicory flour in bakery to benefit from its nutritional and functional qualities. Industrial chicory bitterness is mainly due to the presence of STLs [8–10]. These compounds represent on average 0.42% of the dry weight of the roots, making them the major specialized metabolites found in the root [11]. They belong to the class of guaianolides and are: lactucin, lactucopicrin, 8-deoxylactucin, their 15-oxalated and 15-glycosylated forms, and their derivatives 11(S),13-dihydro [12–14]. The biosynthesis of the guaianolides starts with the conversion of farnesyl-pyrophosphate (FPP) into germacrene A by germacrene A synthase (GAS) [15]. Four copies of *CiGAS* gene were identified in the industrial chicory genome, including a single copy of *CiGAS-long* form and three expressed copies of *CiGAS-short* form [16]. Then cytochrome P450 enzymes germacrene A oxidase (GAO) and costunolide synthase (COS) act consecu-

tively to produce costunolide [17–20]. Recently, a new step of this biosynthetic pathway was revealed with the identification of the kauniolide synthase (*KLS*) gene cluster involved in the conversion of costunolide to kauniolide [21].

Genome editing approaches using CRISPR/Cas9 have developed rapidly in recent years in several plant species. They greatly accelerated gene function analysis or crop improvement to generate new varieties in a shorter time [22–24]. Its use allows to rapidly test strategy to reduce the bitterness of chicory in order to make it more acceptable for its use as a functional ingredient. In *C. intybus*, the CRISPR/Cas9 editing system was first successfully used by Bernard *et al*. [25] using two methods of transformation: *Rhizobium rhizogenes*-mediated transformation and protoplast transfection. Thereafter, the method was used to validate the function of genes involved in the biosynthesis of phenolamide in chicory pollen grains [26]. In parallel, CRISPR/Cas9 technology has been used in several studies to modify the level of STLs in *C. intybus*. First, De Bruyn *et al*. [27] edited *CiGAS*, *CiGAO* and *CiCOS* genes of *C. intybus* var. *foliosum* through PEG-mediated protoplast transfection but the effects on the chemical phenotype was not described. Then Cankar *et al*. [28] edited *CiGAS* genes of *C. intybus* var. *sativum* using the same editing transfection system and showed a strong reduction in the content of lactucin, lactucopicrin, 8-deoxylactucin and their oxalated forms. These results confirmed those of a previous study in which the *CiGAS* genes were silenced by an amiRNA approach [29]. Recently, Cankar *et al*. [21] showed that knockout (KO) of *KLS* genes led to a nearly complete elimination of the major chicory STLs and their oxalate forms in leaves and roots of genome-edited chicories. They also investigated the presence of intermediates in the STL pathway and showed that there was no accumulation of compounds in the leaves, in contrast to the taproot where costunolide, its conjugates and germacrene A acid were detected. Despite these works, no sensory analysis was correlated with these results to determine the effect of STLs reduction on bitterness perception.

In the present study, in order to induce a modulation in STL content, industrial chicories ChicBitter002, cultivated for their roots, were edited with CRISPR/Cas9 technology using *R. rhizogenes*-mediated transformation. The *CiGAS-short* form and *CiGAO* genes, involved in the STL pathway were targeted. In addition, to link the STL content to the bitterness level, a sensory analysis was carried out on roots of several field-grown chicories. A linear correlation was established between the bitterness score associated with each of these chicories and their STL content. A bitterness scale was then generated and used to evaluate the bitterness of the genome-edited chicories based on their STL content. The involvement of the different targeted genes in the synthesis of STLs was also discussed.

2. Materials and Methods

2.1 Plant Material

Field-grown chicories R01 to R13, furnished by Florimond-Desprez, are a breeding material chosen for their variability in the STL content based on the dosage of STLs conducted in 2018 and 2019 (unpublished data). They have been cultivated in 2020 (Coutiches, France) for their roots by Florimond-Desprez SA (Cappelle-en-Pévèle, France). As chicory is allogamous and not able to form stable lineages, each chicory R01 to R13 is a population sharing a common gene pool. For the sensory analysis, each field-grown chicory was furnished as a sample originating from a pool of all roots of three field plots. ChicBitter002, is an *in vitro*-propagated industrial chicory clone, which genome has been sequenced by the Gentiane platform (INRAE, Clermont-Fermond, France) for Florimond Desprez SA (Cappelle-en-Pévèle, France). The clone ChicBitter002 was used in a wild-type form (Ctrl) or transformed by *R. rhizogenes* (HR lines).

2.2 Identification of CiGAS and CiGAO Genes

Several *CiGAS* gene candidates were identified in the industrial chicory genome ChicBitter002 with Blastp or tBlastn algorithms using the *germacrene A synthase short form* gene (Uniprot accession: Q8LSC2) and the *germacrene A synthase long form* gene (Uniprot accession: Q8LSC3) from Bouwmeester *et al*. [30] as queries and with 26,367 'high probability' predictive gene models from the Chicbitter002 sequence (unpublished database, Florimond Desprez SA, Cappelle-en-Pévèle, France) and 31,631 predictive gene models from a witloof chicory genome (Cargese program) or their complete genome sequences as subjects. Nucleotide sequences of significant hits were aligned to the Chicbitter002 sequence using GMAP and their positions were compared to corresponding positions of predictive gene models of recently sequenced Asteraceae, such as *Lactuca sativa*, *Cynara cardunculus* var. *scolymus*, *Artemisia annua* and transcript sequences from unpublished RNA-seq data in industrial and witloof chicories [31–34]. The sequence of the *CiGAO* gene (Uniprot accession: D5JBW8) was used to identify candidates using the same procedure as for *CiGAS* genes. The *CiGAS* gene candidates were used as queries in a tBlastn procedure by comparing them to the BAC clone sequences previously published [16]. Multiple protein sequence alignment from the whole set of *CiGAS* sequences was carried out using MAFFT (**Supplementary Fig. 1**) [35].

2.3 CRISPR/Cas9 Vector Construction

Guide RNAs (sgRNAs) for *CiGAS-short* form and *CiGAO* genes were designed using the software CRISPOR (crispor.tefor.net) based on their GC content and their Doench score [36]. Two sgRNAs were defined for each gene except the gene *CiGAS-S5* for which only one target could be defined (**Supplementary Table 1**). Poten-

tial off-targets were checked with the tool Cas-OFFINDER and verified on the chicory ChicBitter002 genome (unpublished sequence, Florimond Desprez SA, France) to avoid the disruption of off-target gene coding region [37]. Two binary expression vectors for CRISPR/Cas9 were constructed as described previously [25,38]. Briefly, each sgRNA was inserted by digestion/ligation into an intermediate plasmid pKanCiU6-1p-sgRNAscaffold, which includes *C. intybus* U6-1p promoter and a sgRNA scaffold. Four vectors containing the cassette "CiU6-1p-Guide-sgRNAscaffold" were obtained. Adaptors for Golden Gate restriction/ligation method were added to the end of each cassette by PCR using PrimeStar HS polymerase (Takara Bio Europe, St-Germain-en-Laye, France) using primers GG1-F & GG1-R (**Supplementary Table 1**) for plasmid containing CiGAS_T1 and CiGAO_T1 guide and primers GG2-F & GG2-R (**Supplementary Table 1**) for plasmid containing CiGAS_T2 and CiGAO_T2 guide. PCR products were purified with NucleoSpin Gel and PCR Clean-up kits (Macherey-Nagel, Düren, Germany) and were cloned in pYLCRISPR/Cas9P$_{35s}$-B (Addgene plasmid #66190, Cambridge, MA, USA) by Golden Gate restriction/ligation method. Final plasmids pYLCRISPR-GASshort containing CiGAS_T1 and CiGAS_T2, and pYLCRISPR-GAO containing CiGAO_T1 and CiGAO_T2 were obtained.

2.4 Generation of Chicory Transformed Plants

In vitro plants of *C. intybus* L. ChicBitter002 clone were used in this study. Plants were maintained on H10 solid medium as previously described [25]. Wild-type *R. rhizogenes* 15834 strains (provided by Marc Buée, INRAE, Nancy, France) were transformed with pYLCRISPR-GASsh or pYLCRISPR-GAO by electroporation. Wild-type (WT) strains or transformed strains of *R. rhizogenes* were used to generate hairy root (HR) lines from ChicBitter002 chicory. HRs were obtained and maintained in hormone-free media as previously described and spontaneous shoots regenerated from HRs were transfered in H10 medium [25]. A selection of HR mutant lines and control plants were grown in pots for a period of 3 months in a S2 greenhouse. Genomic DNA from HRs was extracted using NucleoSpin Plant II kit (Macherey-Nagel, Düren, Germany) using the manufacturer's protocol. *RolB* specific primers were used to confirm *R. rhizogenes* infection, and *Cas9* presence was checked by PCR using primer pair C9-F and C9-R (**Supplementary Table 1**).

2.5 Genotyping and Sequencing of Edited HR Chicory Plants

Genotyping of HRs was realized to select mutant lines. Primer pairs GAO and S1 to S5 (**Supplementary Table 1**) labelled with different fluorescent dyes (6-Fam or Hex) were designed to amplify the sequences of *GAO* gene or each potentially expressed copy of *CiGAS-short* form gene. The PCR assay was performed in a volume of 15 µL containing 1X PCR buffer with 2 mM $MgCl_2$, 100 µM of each dNTP, 0.2 mg·mL^{-1} BSA, 133 nM of each primer, 0.3 U Taq polymerase (Applied Biosystems, Foster City, CA, USA) and 2 ng of template DNA. A touchdown (TD) procedure was applied: 5 min denaturation at 94 °C, followed by (a) 5 cycles of 30 sec at 94 °C, TD 30 sec at 60 °C (–1 °C per cycle), 30 sec at 72 °C and (b) 35 cycles of 30 sec at 94 °C, 30 sec at 55 °C, 30 sec at 72 °C, before 10 min of extension at 72 °C. The PCR products were analyzed on a 3100 Avant Genetic Analyzer capillary sequencer (Applied Biosystems). Genotyping data were automatically collected and analyzed (GeneMapper Software v3.5, Applied Biosystems, Foster City, CA, USA). Sequences of mutated genes were obtained by Sanger sequencing using S1 to S5 primers pairs for *CiGAS-short* form genes and one GAO primer pair for *CiGAO* gene (GATC service, Eurofins Genomics, Ebersberg, Germany).

2.6 Determination of the STL Content in Chicory Materials

Roots of edited chicories (n = 3 for each HR lines), WT 3-month-old chicories (n = 4) and field-grown chicories (R01 to R13) taproots were freeze-dried and powdered. Hundred mg of powder were extracted with 1 mL of water (LC-MS grade) under agitation (1400 rpm; Eppendorf ThermoMixer C, Hamburg, Germany) for 15 min at 35 °C. The supernatants were collected by centrifugation (15 min, 4 °C, 21,000 g) and boiled for 10 min at 100 °C under agitation (600 rpm). All aqueous extracts were finally filtered through a 0.45 µm PP Whatman UNIFILTER microplate (Cytiva, VWR, Rosny-sous-Bois, France) and transferred to vials for LC-UV analysis. STLs content was determined using an Ultimate 3000RS system equipped with an LPG-3400RS pump, a WPS-3000TRS autosampler and a DAD-3000RS (Thermo Fisher Scientific, Waltham, MA, USA). Chromatographic separation was achieved on an Uptisphere Strategy PHC4 column (3 µm, 150 × 3 mm: Interchim, Montluçon, France) at 50 °C with 0.1% (v/v) formic acid in acetonitrile (B) and 0.1% (v/v) formic acid in water (A) as mobile phases at flow rate of 0.6 mL·min^{-1}. The gradient elution was as follows: isocratic at 10% B for 2.5 min; linear from 2 to 30% B in 40 min; linear from 30 to 85% B in 1 min; isocratic at 85% B for 2 min; linear from 85 to 10% B in 1 min; and isocratic at 10% B for 4 min. The injection volume was 5.0 µL. Fifteen STLs compounds were profiled based on this method and determined by LC-QTOF-HRMS analysis. Total STLs content, in all the analyses, was expressed as the sum of the peak areas of these 15 compounds, present in the UV chromatogram at 254 nm.

2.7 LC-QTOF-HRMS Analysis of Sesquiterpene Lactones

STL compounds were identified using an Ultimate 3000RS system equipped with a DAD-3000RS (Thermo Fisher Scientific, Waltham, MA, USA), interfaced with a high-resolution quadrupole time-of-flight mass spectrometer and equipped with an ESI source (Impact II, Bruker

Daltonik GmbH, Bremen, Germany). One gram of freeze-dried powdered chicory root from the pool of field-grown chicories (R01-R13) was extracted with 10 mL of water (LC-MS grade) under agitation (1000 rpm) for 15 min at 35 °C. After centrifugation (15 min, 4 °C, 21,000 g) , the supernatant was immediately evaporated, and the residue was re-suspended in 2% acetonitrile (v/v) containing 0.1% formic acid (v/v) and passed through a 0.2 µm hydrophilic PTFE syringe filter (Merck, Darmstadt, Germany) and transferred to a vial for LC-MS analysis. Chromatographic separation was achieved on an Uptisphere Strategy PHC4 column (2.2 µm, 150 × 3 mm; Interchim, Montluçon, France) at 30 °C with 0.1% (v/v) formic acid in acetonitrile (B) and 0.1% (v/v) formic acid in water (A) as mobile phases at flow rate of 0.5 mL·min^{-1}. The gradient elution was as follows: isocratic at 2% B for 1 min; linear from 2 to 50% B in 32 min; linear from 50 to 100% B in 5 min; isocratic at 100% B for 7 min; linear from 100 to 2% B in 5 min; and isocratic at 2% B for 7 min. The injection volume was 2.0 µL. The mass spectrometer was operated in the positive electrospray ionization mode at 4.5 kV with the following parameters: nebulizer gas (N_2), 2.4 bars; dry gas (N_2), 10.0 mL·min^{-1}; dry temperature, 250 °C; collision cell energy, 10.0 eV; end plate offset, 500 V. Sodium formate cluster ions were applied for instrument mass calibration and for re-calibration of individual raw data files. The acquisition was performed in full scan mode in the 90–1200 m/z range with an acquisition rate of 1 Hz and with mass accuracy <5 ppm. Software Compass otofControl 4.1 (Mount Vernon, NY, USA) was used for the operation of the mass spectrometer and for data acquisition, and data were processed by the software Compass DataAnalysis 4.4 (Bruker Daltonik GmbH, Bremen, Germany).

2.8 Construction of Bitterness Reference Scale

The sensory evaluation was performed at the agrifood platform of Polytech Lille (University of Lille, France) in laboratory rooms according to ISO 8589:2007. The panel was constituted from volunteers recruited among the personnel of the University of Lille and trained in accordance with ISO 8586:2012. Panelists were first selected based on their ability to recognize bitterness, sweetness, saltiness, and sourness. Four series of different concentrations of taste solutions in water were prepared by dilution of stock solutions as followed: bitter with caffeine (concentrations: 0.27, 0.35, 0.46, 0.59 g·L^{-1}), sweet with sucrose (concentrations: 12, 24, 48, 96 g·L^{-1}), salty with sodium chloride (concentrations: 2, 4, 8, 16 g·L^{-1}) and sour with citric acid (concentrations: 0.6, 1.2, 2.4, 4.8 g·L^{-1}); the lowest dilutions corresponding to the perception threshold for the substance evaluated. Participants were first asked to identify the four tastes for each concentration, as well as pure water blank. Subsequently, they ranked the different concentrations using a 10 cm horizontal line. Each test was repeated twice. Fourteen panelists were retained and to familiarize themselves with the bitterness of chicory, additional training with solutions of free STLs was performed. The six solutions were prepared according to the concentration range of free STLs from 0.62 mM to 0.019 mM, from a serial dilution of chicory root extract enriched in free STLs [39]. The solutions, including a pure water solution as a blank, were presented in plastic glasses accompanied with a plastic drop-pipette to pour solution into the mouth. Participants were invited to taste 7 mL of each dilution with two replications. They were asked to rate the different solutions according to the bitterness intensity using a 7-point ranking; the rank 1 corresponding to the least intense perceived concentration. At the end, twelve panelists were selected for the quantitative sensory evaluation. Finally, freeze-dried root powders from field-grown chicories (R01 to R13) were evaluated by the panel for their bitterness intensity according to ISO 6658:2017. The samples were served on plastic cups labeled with random three-digit numbers. The panelists were asked to rank the samples using a 10 cm horizontal line and evaluation was repeated twice in two sessions. The bitterness reference scale was constructed based on a simple linear regression model plotting bitter taste in function of relative total STL content. The bitterness degree of the samples from the roots of edited-chicory plants was then predicted by fitting their total STL content by means of the regression curve equation.

2.9 Statistical Analysis

An ANOVA and Student's *t*-test were performed to determined panelist performance (discrimination ability, consensus in notation, repeatability) and to test for bitterness differences between the chicory root samples from the field-grown chicories (R01-R13). Comparison of STL content of edited and WT 3-month-old chicories and assessment of the differences in panelist perception of bitter taster were tested by non-parametric ANOVA (Kruskal-Wallis) followed by post-hoc uncorrected Dunn's test or post-hoc paired Wilcoxon, respectively. The assumptions of normality, homogeneity of variance, and independence were checked by Shapiro-Wilk test, Bartlett test, and Durbin-Watson test, respectively. The bitterness correlation with STL content was established by Pearson's correlation test. The significance threshold was set to 0.05 for all statistical tests. All analyses were done with R Statistics 4.0.3 (r-project.org) and GraphPad Prism 9 software (Windows Inc., San Diego, CA, USA).

3. Results

3.1 Identification of CiGAS and CiGAO Genes

The genome of the industrial chicory ChicBitter002 was searched to identify *CiGAS* and *CiGAO* genes putatively involved in the STL pathway. A single copy of *CiGAO* gene was highlighted in the genome of ChicBitter002 (**Supplementary Fig. 2**). For *CiGAS* genes, quite similar data to Bogdanović *et al*. [16] were found with one

gene for *CiGAS-long* form and five gene copies of *CiGAS-short* form. Sequence analysis confirmed the existence of *GAS-S1*, *GAS-S2*, *GAS-S3* and *CiGAS-S4b* but *CiGAS-S4a* was not found. However a new *CiGAS-short* form candidate copy, named *CiGAS-S5*, was highlighted (Fig. 1). Sequence analysis confirmed that copies *CiGAS-S1*, *CiGAS-S2*, *CiGAS-S3* and *CiGAS-S5* could be expressed with a complete 7-exons structure and that copy *CiGAS-S4b* has a retrotransposon into the exon 3 that could hamper its expression. The five copies were identified at two different locations in the genome. On the one hand, *CiGAS-S1*, *S2* and *S3* were located on the same contig (contig_403_pilon) so they can be considered as three distinct genes but physically close on the chicory genome. Indeed, the *CiGAS-S1* and the *CiGAS-S3* are separated by ~85,000 bp, with *CiGAS-S2* in between (**Supplementary Fig. 3**). It can also be noted that *CiGAO* gene is present on the same contig as the three *CiGAS-short* form genes, and ~85,000 bp away from the *CiGAS-S3* copy (**Supplementary Fig. 3**). On the other hand, *CiGAS-S4b* and *S5* also shared the same contig (contig_1740_pilon) and are ~76,000 bp apart. Based on these results, 4 copies of *CiGAS-short* can be considered as putatively expressed : copies *S1*, *S2*, *S3* and *S5* (Fig. 1). Since the copy *S4b* was presumably not expressed due to the presence of a retrotransposon, it was not targeted.

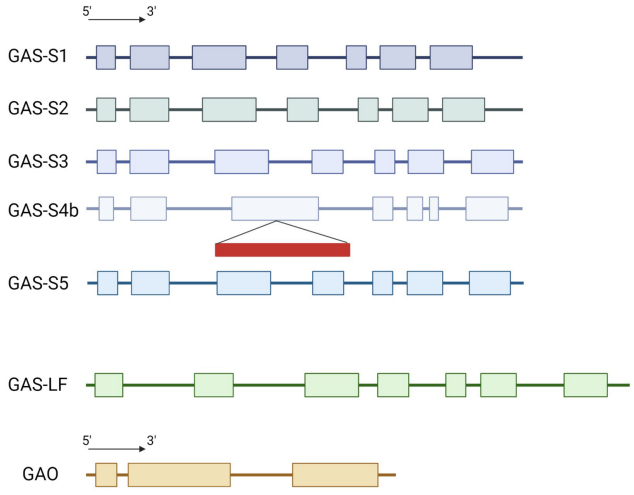

Fig. 1. Schematic representation of the structure for *CiGAS* and *CiGAO* genes. GAS-S stands for the *CiGAS-short* form and GAS-LF stands for the *CiGAS-long* form. The exons and introns lengths are proportional to the nucleotide sequences of the genes. Colored box: exon, line: intron, red rectangle: retrotransposon.

3.2 Targeting of CiGAS and CiGAO Genes

The objective was to perform a KO of the 4 putatively expressed copies of *CiGAS-short* form and of the *CiGAO* gene. In order to design sgRNAs for the four putatively expressed copies of *CiGAS-short* form, the homology of these genes was investigated by comparing their exonic sequences using ClustalOmega tool [40]. All the putatively expressed copies (*CiGAS-S1* to *S5*) are relatively close since they share between 86 to 97% identity. *CiGAS-S1* and *CiGAS-S2* sharing the highest identity (**Supplementary Fig. 4**). The newly identified *CiGAS-S5* copy shares 86–93% identity with the other copies, confirming its link to the multiple copies of *CiGAS-short* form (**Supplementary Fig. 4**). The high level of homology between the potentially expressed copies of *CiGAS-short* form did not allow specific targeting of each copy, but it was possible to design sgRNAs in the third exon to target all copies simultaneously. Since a single copy of *CiGAO* was identified, two sgRNAs were designed at the beginning of the first exon in order to disrupt the gene function.

The use of the CRISPOR tool to determine the potential targets gave a list of sgRNAs that were then analyzed using the Cas-OFFINDER tool to search for potential off-targets. The results were checked on the ChicBitter002 genome, to avoid selecting sgRNAs with off-targets in gene coding regions. Finally, two sgRNAs were identified in the first exon of *CiGAO* and two sgRNAs were identified in the third exon of *CiGAS-S1*, *CiGAS-S2* and *CiGAS-S3*. For the *CiGAS-S5* copy, a single sgRNA common to the other copies was defined (**Supplementary Table 1**). The *GAS-long* gene was not targeted because it has been demonstrated to have minimal impact on STLs production in chicory roots [28].

3.3 Identification of the CRISPR/Cas9 Mutagenesis Events and Characterization of the HR Mutant Lines

Chicory leaves of 14-day-old vitroplants were infected with *R. rhizogenes* strain 15834 transformed with the binary vectors pYLCRISPR-GASshort or pYLCRISPR-GAO. Two weeks after *R. rhizogenes* transformation, selection of HRs based on their phenotype was carried out. Eighty-five HR lines were collected with 54 lines potentially transformed with the vector targeting the *CiGAS-short* form genes and 31 lines potentially transformed with the vector targeting the *CiGAO* gene. After verification of the presence of the T-DNA of *R. rhizogenes* and the integration of the binary vector, the number of transformed lines was reduced to 43 for *CiGAS-short* and 30 for *CiGAO* (Table 1). As integration of the binary vector is not always synonymous of mutation, a preliminary sorting of the mutants was performed by genotyping. Indeed, the action of CRISPR/Cas9 can generate a variety of mutated alleles and because multiple copies of *CiGAS-short* were highlighted, it is possible that all copies are not mutated at the same time. Then, a Sanger sequencing of the mutant HR lines identified by genotyping was performed to obtain the sequences of the mutants. Out of the 43 lines transformed with pYLCRISPR-GASshort, analysis of the mutations revealed 6 lines with one copy of *CiGAS* gene mutated, 4 lines with two mutated copies and 3 lines with three mu-

Table 1. Overview of the mutation events in chicory transformed with *R. rhizogenes* with different constructs.

Genes		*CiGAS-short*	*CiGAO*	Control
Initial number of HR lines		54	31	6
Number of lines with binary vector	Total	43	30	6
	WT	30	24	6
	1 copy mutated (KO)	6 (3)	6 (2)	0
	2 copies mutated (KO)	4 (1)	nc	nc
	3 copies mutated (KO)	3 (1)	nc	nc
	4 copies mutated (KO)	0 (0)	nc	nc
Mutation frequency		30.2%	20.0%	0.0%
Gene KO frequency		11.6%	6.6%	0.0%

HR, Hairy Root; WT, Wild-Type lines; KO, Knock-Out due to the onset of premature stop codon; nc, not concerned. Mutation frequency was calculated by dividing the sum of mutant lines by the total of lines with binary vector. Gene KO frequency was calculated by dividing the sum of (KO) by the total of lines with binary vector.

Table 2. Summary of mutations found in hairy root lines for each target of the different copies of *CiGAS-short* and for *CiGAO* genes.

	CiGAS-short							***CiGAO***	
	S1		**S2**		**S3**		**S5**		
Line	T1	T2	T1	T2	T1	T2	T1	T1	T2
HR1	–3/–3	+1/+1	WT/WT	WT/WT	WT/WT	WT/WT	WT/WT	WT/WT	WT/WT
HR2	WT/WT	–5/+1	WT/WT	WT/WT	WT/WT	WT/WT	WT/WT	WT/WT	WT/WT
HR3	WT/WT	WT/–41	WT/WT	WT/WT	WT/WT	WT/WT	WT/WT	WT/WT	WT/WT
HR4	WT/WT	WT/WT	+1/+1–45	+1/–45	WT/WT	WT/WT	WT/WT	WT/WT	WT/WT
HR5	WT/WT	WT/WT	WT/+1	WT/–6	WT/WT	WT/WT	WT/WT	WT/WT	WT/WT
HR6	WT/WT	WT/WT	WT/+1	WT/–6	WT/WT	WT/WT	WT/WT	WT/WT	WT/WT
HR7	WT/–31	WT/–31	WT/WT	WT/–10	WT/WT	WT/WT	WT/WT	WT/WT	WT/WT
HR8	WT/–29	+1/–29	WT/WT	+1/–1	WT/WT	WT/WT	WT/WT	WT/WT	WT/WT
HR9	WT/WT	WT/–5	WT/+1	WT/–6	WT/WT	WT/WT	WT/WT	WT/WT	WT/WT
HR10	WT/WT	WT/–5	WT/+1	WT/–6	WT/WT	WT/WT	WT/WT	WT/WT	WT/WT
HR11	WT/WT	WT/–10	WT/WT	WT/–3	WT/WT	WT/WT	WT/–7	WT/WT	WT/WT
HR12	WT/–29	+1/–29	WT/WT	+1/+1	WT/WT	WT/WT	WT/WT	WT/WT	WT/WT
HR12*	WT/–29	+1/–29	WT/WT	+1/+1	WT/WT	WT/WT	+1/–1	WT/WT	WT/WT
HR13	WT/WT	WT/+1	WT/WT	+1/–41	+1/–3	WT/WT	WT/WT	WT/WT	WT/WT
HR14	WT/WT	WT/WT	WT/WT	WT/WT	WT/WT	WT/WT	WT/WT	WT/WT	WT/+1
HR15	WT/WT	WT/WT	WT/WT	WT/WT	WT/WT	WT/WT	WT/WT	–12/–22	WT/–7
HR16	WT/WT	WT/WT	WT/WT	WT/WT	WT/WT	WT/WT	WT/WT	WT/+1	WT/WT
HR17	WT/WT	WT/WT	WT/WT	WT/WT	WT/WT	WT/WT	WT/WT	WT/–22	WT/–6
HR18	WT/WT	WT/WT	WT/WT	WT/WT	WT/WT	WT/WT	WT/WT	+1/–8	WT/WT
HR19	WT/WT	WT/WT	WT/WT	WT/WT	WT/WT	WT/WT	WT/WT	WT/WT	–7/–7

Mutation leading to a change in the coding frame are in red. Larger deletion between the two targets (inter-guide deletion) are underlined. HR12* correspond to the line HR12 but with an additional mutation as a result of the CRISPR/Cas9 action. T1, Target 1; T2, Target 2; WT, Wild-Type; +, insertion; -, deletion.

tated copies, resulting in a mutation rate of 30.2% (Table 1). HRs transformed with pYLCRISPR-GAO showed a mutation rate of 20% with 6 mutated lines out of 30 transformed lines (Table 1).

As the chicory genome is diploid, the CRISPR/Cas9 system can induce two types of mutations. On the one hand, a monoallelic mutation could occur. In such case, one allele is mutated and the other remains wild-type (heterozygous) leading to an absence of full KO of the targeted gene. On the other hand, editing could generate a biallelic mutation where both alleles are mutated, either with the same mutation on each allele (homozygous), or with a different mutation (also heterozygous) that can lead to a KO of the gene (Table 2 and Fig. 2). Most of the identified mutations were small insertions of one nucleotide (HR1, HR2, HR4, HR5, HR6, HR8, HR9, HR10, HR12, HR13, HR14, HR16, or

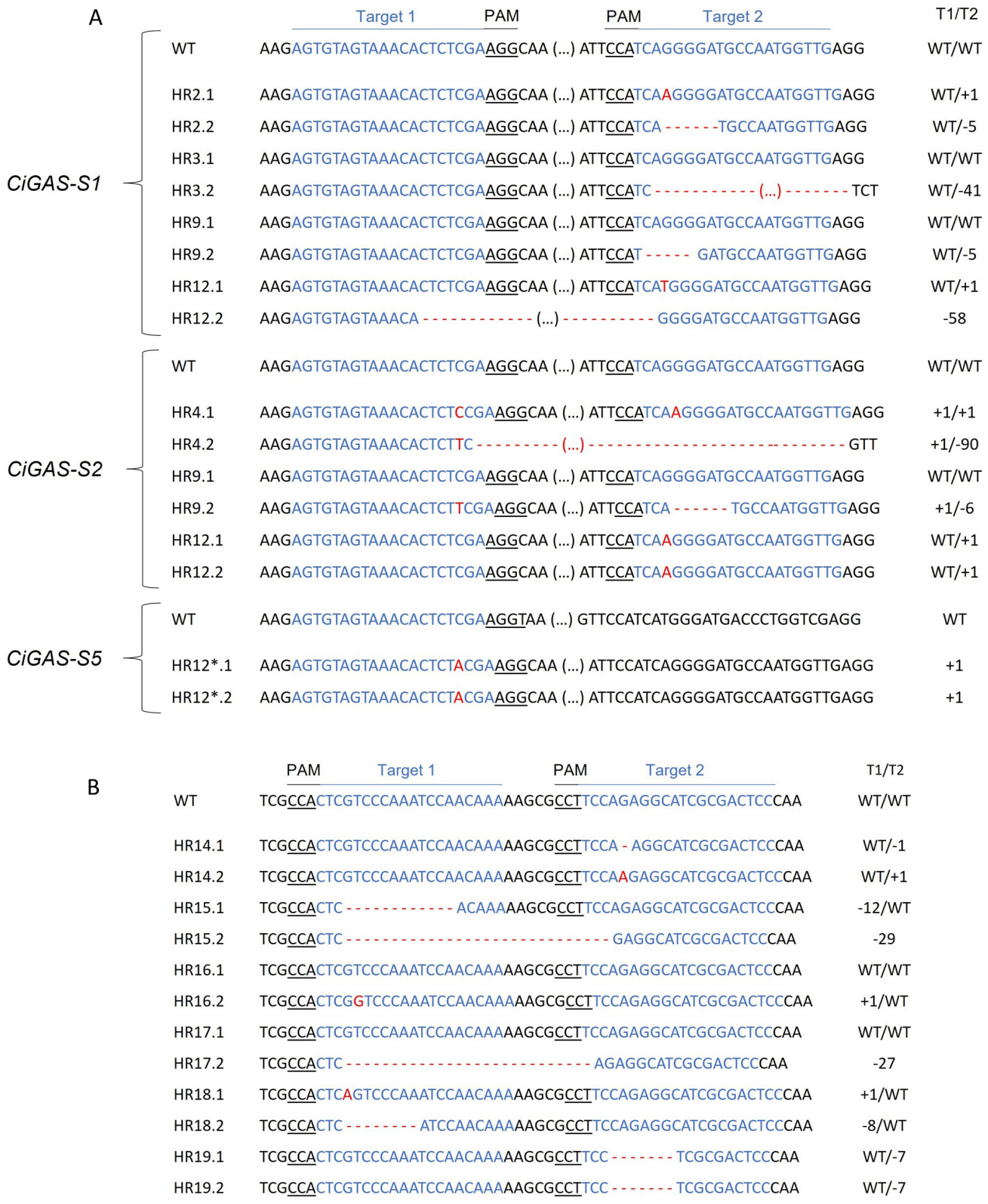

Fig. 2. Examples of sequences obtained by CRISPR/Cas9 editing. (A) Sequence analysis of the two alleles of hairy root lines transformed with pYLCRISPR-GASshort construct. (B) Sequence analysis of the two alleles of hairy root lines transformed with pYLCRISPR-GAO construct. The target sequences are in blue, and the PAM sequences are underlined. Mutations are highlighted in red and the changes in the nucleotide sequences are shown on the right of each allele. HR.x.x, Hairy Root x allele x; WT, Wild-Type; T1, Target 1; T2, Target 2; +, insertion; -, deletion.

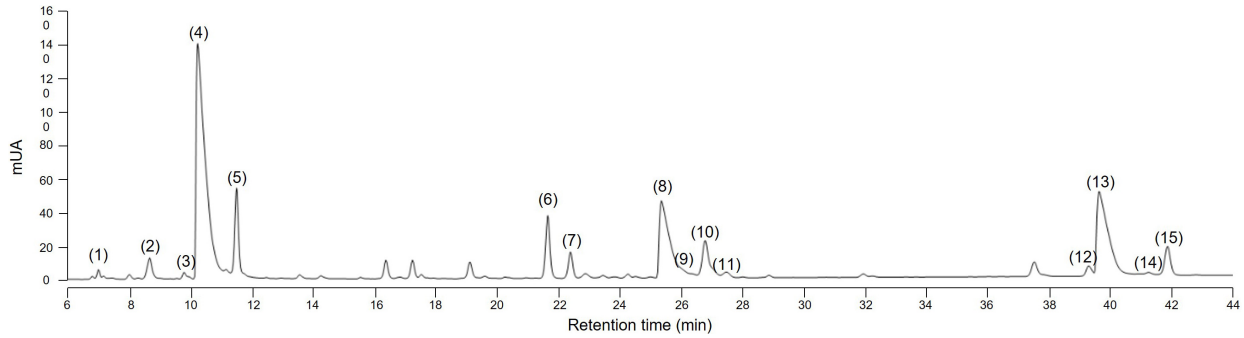

Fig. 3. HPLC chromatogram at 254 nm of sesquiterpene lactones in root of the wild-type chicory ChicBitter002. (1) 11(S),13-dihydrolactucin-15-glycoside (DHLc-gly); (2) 11(S),13-dihydrolactucin-15-oxalate (DHLc-ox); (3) 11(S),13-dihydrolactucin (DHLc); (4) Lactucin-15-oxalate (Lc-ox); (5) Lactucin (Lc); (6) 8-deoxylactucin-15-glycoside (dLc-gly); (7) 11(S),13-dihydro-8-deoxylactucin-glycoside (DHdLc-gly); (8) 8-deoxylactucin-15-oxalate (dLc-ox); (9) 11(S),13-dihydro-8-deoxylactucin-15-oxalate (DHdLc-ox); (10) 8-deoxylactucin (dLc); (11) 11(S),13-dihydro-8-deoxylactucin (DHdLc); (12) 11(S),13-dihydrolactucopicrin-15-oxalate (DHLp-ox); (13) Lactucopicrin-15-oxalate (Lp-ox); (14) 11(S),13-dihydrolactucopicrin (DHLp); (15) Lactucopicrin (Lp). Identity of the molecules was confirmed by mass spectrometry.

HR18) or small deletions of less than 10 nucleotides (HR1, HR2, HR5, HR6, HR7, HR9, HR10, HR11, HR13, HR15, HR17, HR18, or HR19), but in some case, larger deletions were found between the two targets T1 and T2 as shown for HR4, HR7, HR8, HR12, HR12*, HR15 and HR17 (Fig. 2). Some mutations cause a change in the coding frame leading to the KO of a gene or a gene copy and the potentially premature termination of protein translation. This type of event was observed for 5 HR lines mutated on *CiGAS-short* genes: 3 lines mutated on a single copy (HR1, HR2 and HR4), 1 line mutated on two copies (HR8) and 1 line mutated on 3 copies (HR12*); and in 2 HR lines mutated on *CiGAO* (HR18 and HR19) resulting in a gene KO frequency of 11.6% and 6.6% respectively (Tables 1,2). The *R. rhizogenes*-mediated transformation is a stable transformation, meaning that the T-DNA is inserted into the plant genome. As a result, the *Cas9* gene is integrated into the genome of HR lines and can continue to exert its action and cause mutations. This case can be observed for the lines HR12 and HR12*. Originally, HR12 had a mutation on both *CiGAS-S1* and *CiGAS-S2* copies but after a few months, an additional mutation appeared on the *CiGAS-S5* copy.

For the rest of the paper, we will focus on only few mutants (HR2, HR3, HR9, HR12, HR12*, HR16 and HR18) to see which mutation event can affect the STL production.

3.4 Presence of Different STL Forms in the Chicory Materials

Several methods to extract sesquiterpene lactones from chicory roots have been described using various solvents, but a method more representative of physiological conditions of human consumption was desired, since the objective of our project is to use industrial chicory root as functional eaten ingredient. Using water, an aqueous extract rich in sesquiterpene lactones was obtained with 15 compounds detected at a wavelength of 254 nm in the WT chicory ChicBitter002: lactucin, 8-deoxylactucin, lactucopicrin and their 11(S),13-dihydro derivatives, in addition of their oxalated forms and some glycosylated forms (Fig. 3). The same extraction method was used for the field-grown chicories used in sensory analysis and their STL composition was compared to that of the WT chicory ChicBitter002 “Ctrl” grown under controlled conditions (*in vitro* then in greenhouse). Fifteen identified compounds are present in all chicories, but a quantitative disparity was observed between the field-grown chicories (R01-R13) and the chicory “Ctrl” (Fig. 4A). As a general trend, the lactucin-15-oxalate and 8-deoxylactucin-15-glycoside content appeared to be higher in “Ctrl” plants compared with the field-grown chicories, whereas the different forms of 11(S),13-dihydrolactucin were in lower quantities. These differences could be due to the growing conditions or genotypic. However, if the distribution of STL content was examined according to the lactucin-like, 8-deoxylactucin-like, and lactucopicrin-like groups, the difference between chicories would fade, as would the total STL content (Fig. 4B). In the following analyses, the STL content of the chicories were therefore compared by considering their total STLs obtained by the sum of the 15 compounds detected in Fig. 3. This is based on the hypothesis that taking into account all STLs is a good indicator of bitterness. Indeed, the different studies on chicory have not clearly established a compound more involved than another in bitterness and when consuming chicory-based products, the consumer doest not taste the STLs separately in his mouth but perceives them as a whole and detects a bitter taste.

3.5 Analysis of the Total STLs Content in Edited Plants Regenerated from HR Lines

A particularity of chicory hairy root lines is that they are able to regenerate spontaneous shoots. Thus,

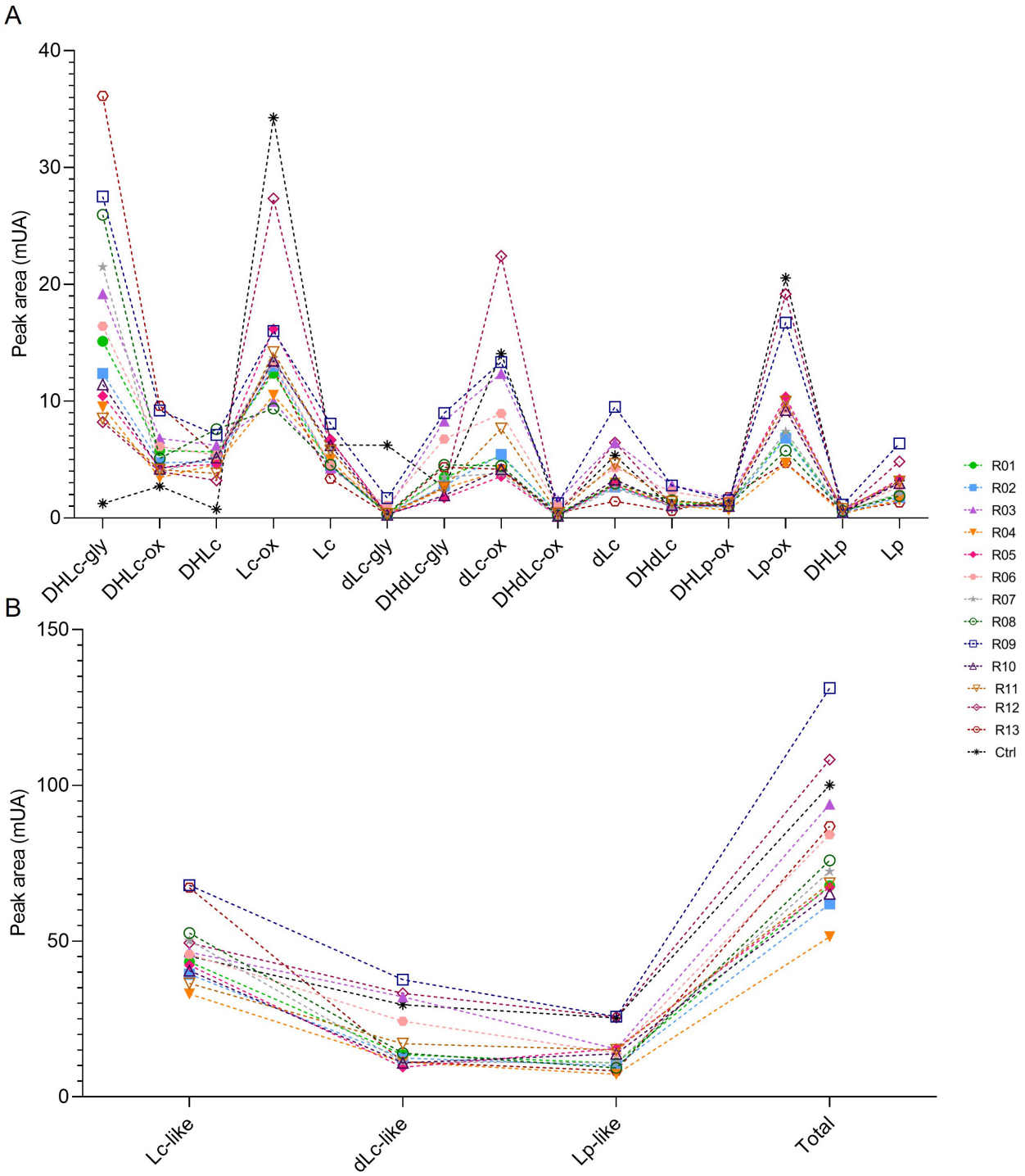

Fig. 4. STL content in roots of chicories according their peak area (mUA) obtained by HPLC analysis. (A) represents the distribution of each STL into the roots of chicories. (B) represents the STL levels of each chicories according to their structural group (Lc-like, dLc-like or Lp-like) and the total STL content of them. R01 to R13 are field-grown chicories used for sensorial analysis. Ctrl is wild-type ChicBitter002 grown under controlled condition. DHLc-gly, 11(S),13-dihydrolactucin-15-glycoside; DHLc-ox, 11(S),13-dihydrolactucin-15-oxalate; DHLc, 11(S),13-dihydrolactucin; Lc-ox, Lactucin-15-oxalate; Lc, Lactucin; dLc-gly, 8-deoxylactucin-15-glycoside; DHdLc-gly, 11(S),13-dihydro-8-deoxylactucin-glycoside; dLc-ox, 8-deoxylactucin-15-oxalate; DHdLc-ox, 11(S),13-dihydro-8-deoxylactucin-15-oxalate; dLc, 8-deoxylactucin; DHdLc, 11(S),13-dihydro-8-deoxylactucin; DHLp-ox, 11(S),13-dihydrolactucopicrin-15-oxalate; Lp-ox, Lactucopicrin-15-oxalate; DHLp, 11(S),13-dihydrolactucopicrin; Lp, Lactucopicrin. Lc-like, sum of all DHLc and Lc forms; dLc-like, sum of all DHdLc and dLc forms; Lp-like, sum of all DHLp and Lp forms; Total, sum of the 15 identified STLs.

from the mutated hairy root lines we were able to regenerate whole plants that were grown in a greenhouse for 3 months. Applying the previously described extraction method, 3-month-old edited chicory roots (n = 3 for each HR lines selected) were analyzed to evaluate the impact of the CRISPR/Cas9 mutation on STL accumulation.

All the analyzed plants were derived from ChicBitter002 clone, with "Ctrl" corresponding to WT plants and "Ctrl_HR" corresponding to *R. rhizogenes*-infected chicory plants with no pYLCRISPR/Cas9P$_{35s}$-B or binary vector pYLCRISPR-GASshort or pYLCRISPR-GAO. Comparison of "Ctrl" and "Ctrl_HR" showed an increase in STL content for *R. rhizogenes*-infected plants (Fig. 5). For the rest of the analysis, the genome-edited chicory lines were compared to "Ctrl_HR". Seven edited-chicory lines were selected for their various mutation events (monoallelic or biallelic, one copy or several mutated copies). Determination of their STL content was carried out to represent the effect of each targeted gene on STL content.

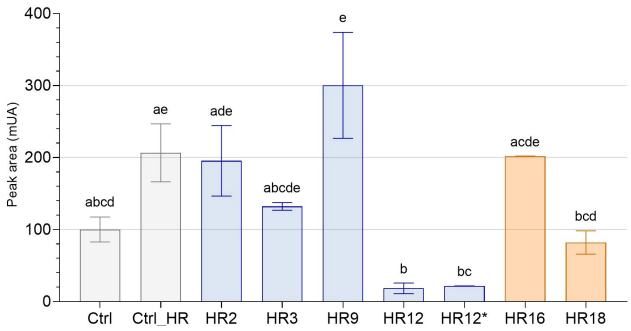

Fig. 5. Total sesquiterpene lactone content of roots of edited chicory regenerated from HR lines by HPLC. The peak areas (mUA) of STLs in the roots of 7 genome edited chicory regenerated from HR lines were analyzed by HPLC and compared to WT chicory lines (Ctrl and Ctrl_HR). Gray bars correspond to control chicory lines, blue bars to *CiGAS-short* edited lines and orange bars to *CiGAO* mutants. The histogram shows the modulation of total STL content (sum of all identified STLs) as a function of mutation on chicory lines. HR12 and HR12* are originally the same hairy root line except that HR12 is mutated for only *CiGAS-S1* and *CiGAS-S2* and HR12* is mutated for *CiGAS-S1*, *CiGAS-S2* and *CiGAS-S5*. The letters indicate significantly different groups according to non-parametric one-way ANOVA and Dunn's post-hoc test ($p < 0.05$).

For the *CiGAS-short* form mutants, 5 lines have been selected: line HR3, a monoallelic mutant which carries a mutation in a single allele of *CiGAS-S1*; line HR2, a heterozygous biallelic mutant for *CiGAS-S1* where editing events induce a change in the coding frame; line HR9, a monoallelic mutant for both *CiGAS-S1* and *CiGAS-S2*, meaning that only one allele is mutated in each of the two genes; line HR12, a heterozygous biallelic mutant for *CiGAS-S1* and a homozygous biallelic mutant for *CiGAS-S2*, meaning there is a change in the coding frame in both of these gene copies (**Supplementary Figs. 5,6**), and line HR12* which is the same as line HR12 except that there is an additional homozygous biallelic mutation for *CiGAS-S5* (**Supplementary Figs. 5–7**). For the *CiGAO* mutants, the 2 selected lines were: line HR16, a monoallelic mutant and line HR18, a heterozygous biallelic mutant with a change of the coding frame of its gene (**Supplementary Fig. 8**). The nucleotide sequences of these lines are described in Fig. 2.

By comparing the total STL content of all these lines with the "Crtl_HR", only three lines showed significative reduction in STL content: HR12, HR12* and HR18. All three share biallelic mutations. All other *CiGAS-short* and *CiGAO* edited lines showed no significant difference in total STL content (Fig. 5). Most are monoallelic mutants, and one is a biallelic mutant only for one copy of the GAS gene (*CiGAS-S1*). Analysis of the STL content of a chicory edited for both alleles of *CiGAS-S2* with a change in the coding frame was also performed and showed no significant difference, but we did not have enough biological replicates to include this result in our data. Regarding the *CiGAS-short* form mutants, both the HR12 and HR12* edited line showed similar profiles where the total STL content was strongly reduced (Fig. 5). This trend was confirmed in total free forms and total oxalate forms of STLs (**Supplementary Fig. 9**).

All these data suggest that the two copies (*S1* and *S2*) of *CiGAS-short* are important for STL production in our plants and must be simultaneously KO to significatively reduce the STL content. The *CiGAO* gene also plays an important role in STL production, but the impact was not as great as for the two copies of *CiGAS-short*.

3.6 Sensory Analysis

Sensory analysis was conducted on the root powder of thirteen field-grown chicory plants (R01 to R13). Each chicory was assigned a bitterness score from 0 to 10, and panelists determined that R09 and R06 were the most bitter chicories (score of 7.83 and 6.13, respectively) and R04 and R13 were perceived as the least bitter chicories (score of 2.21 and 2.49, respectively) (Fig. 6, **Supplementary Table 2)**. First, a correlation between bitterness and each STL of the field-grown chicories was sought to assess whether a single or multiple compounds are involved in bitterness. Of the fifteen STLs identified in this paper, six were not correlated with bitterness: DHLc-gly, DHLc-ox, DHLc, Lc-ox, dLc-ox and, DHLp-ox (**Supplementary Table 3**). The remaining nine were more or less correlated with an Pearson's r ranging from 0.5587 to 0.9223 (**Supplementary Table 3**). Since bitterness cannot be attributed to a single compound, it is assumed that all STLs can be considered indicative of chicory bitterness. Next, the total STL content of each tasted chicory was assessed and a linear regression analysis was carried out to estimate whether the STL con-

tent can be correlated with the bitterness score (Fig. 6). A Pearson correlation allowed to establish a positive correlation (R^2 = 0.46) between the perception of bitterness and the total STL content of chicories, with r = 0.68 (two-tailed *p*-value = 0.0103). Finally, using the resulting linear regression equation, the seven hairy roots edited by CRISPR/Cas9 for *CiGAS-short* and *CiGAO* were assigned theoretical bitterness scores based on their STL content and in ordert to project the hairy root mutants onto the linear regression curve, it was artificially elongated. These data were plotted in Fig. 6. Based on its STL content and its theoretical bitterness score (5.06), the "Ctrl" sample would appear to be more bitter than most field-grown chicories, except for R06 and R09 (Fig. 6). The projection on the correlation curve of the sample "Ctrl_HR", which has a higher STL content than "Ctrl", seems to indicate a high bitterness score (10.10). Therefore, the plant material generated by *R. rhizogenes* infection can initially be considered as very bitter. For the previously analyzed CRISPR/Cas9 edited-chicory lines, most of the theoretical bitterness scores obtained were in the range from 6.75 to 14.41, except the lines HR12, HR12* and HR18, which have theoretical bitterness scores of 1.29, 1.48 and 4.26, respectively (Fig. 6, **Supplementary Table 2**). The lines with the lowest theoretical scores are biallelic mutants for either *CiGAS-S1* and *CiGAS-S2*, or *CiGAS-S1*, *CiGAS-S2* and *CiGAS-S5*, or *CiGAO*, and had low STL contents compared to the "Ctrl_HR" line. The other lines correspond to mutant lines whose STL content did not differ significantly from the "Ctrl_HR" line.

It can be concluded that a complete KO of *CiGAS-S1* and *CiGAS-S2* genes could be sufficient to cause a drastic decrease in STL content that would be reflected in the bitterness score. HR12 and HR12* edited chicory lines almost lost their bitterness with a diminution of 86.35 $\pm$ 0.9% in their theoretical bitterness scores compared to "Ctrl_HR". A full KO of the *CiGAO* gene is also responsible for a low bitterness score since it is 57.8% lower than the control "Ctrl_HR".

4. Discussion

In this work, CRISPR/Cas9 system was successfully used to inactivate *CiGAS-S* and *CiGAO*. As a result, a reduction in STL content and hence presumed bitterness of chicory were obtained.

In the past years, the CRISPR/Cas9 system has been already applied in *C. intybus* to abolish functions of numerous genes. The transformation methods used were either *R. rhizogenes* infection or protoplast transfection, both techniques having advantages and disadvantages [21,25–29]. In this work, *R. rhizogenes*-mediated CRISPR/Cas9 editing was used for its efficiency (mutation frequency of 26%, calculated by the sum of mutant hairy root lines over the sum of total hairy root lines with binary vector), for its ability to generate a large amount of biological material and for the particularity of ChicBitter002 chicory HRs to spontaneously regenerate shoots as described in literature [41,42]. HR-derived plants have a slightly modified chicory phenotype and can flower early without vernalization as it is required for WT plants [29]. The presence of T-DNA of *R. rhizogenes* also seems to activate the production of specialized metabolites as previously observed [43–45]. Indeed, an increase in STL content was observed in "Ctrl_HR" plants when compared to "Ctrl" plants (Fig. 5) and most of the HR mutant lines contained the same amounts of total STLs. However a significant decrease in total STL content was observed when biallelic mutants were obtained (HR12, HR12* and HR18). It is the case of the HR12 line, a biallelic mutant for both *CiGAS-S1* and *CiGAS-S2*, who has a significant decrease in total STL content. These results are consistent with those published by Cankar *et al.* [28] where a decrease in STL content could be observed for biallelic mutants for at least two copies of *CiGAS-S*. However, this decrease in STL content is only significant when all 3 copies of *GAS-S*, or all copies of *CiGAS-S* and *CiGAS-L* are mutated, and not significant when only two copies of *CiGAS-S* are mutated (RN8 and RN10 plants), whereas in our study, the reduction is significant from the moment when two copies of *CiGAS-S* are mutated. Although only few mutants were analyzed in this article, in order to present a panel of mutation events, our results are slightly different from those of Cankar *et al.* [28] and would deserve analyses on a larger number of mutants. In addition, the HR12* line also showed a drastic decrease in total STL content. This line also has a biallelic mutation on both *CiGAS-S1* and *CiGAS-S2* but an additional mutation was observed on *CiGAS-S5*. However, no difference was observed between the total STL content of HR12 and HR12*. The explanation for this result could be that (1) the copy *CiGAS-S5* was expressed at low in our growing conditions or in the root tissue, not compensating for the inhibition of *CiGAS-S1* and *CiGAS-S2*, or was not expressed; or (2) the level of STLs in these HR lines was already very low and the effect of the mutation on *CiGAS-S5* cannot be seen. To discriminate between these hypotheses, it could be considered to analyze the STL content of plants mutated only for *CiGAS-S5* or, on the contrary, mutated for all *CiGAS-short* form except *CiGAS-S5*, or to realize qPCR in order to determine the expression levels of this copy but this could be difficult because the different copies of *CiGAS-short* share high sequence identity. A decrease was also observed for biallelic mutant for *CiGAO* (line HR18), but the impact was not as important as the decrease caused by the biallelic mutation of *CiGAS-S1* and *CiGAS-S2*. Indeed, contrary to our expectations the production of STLs for HR18 line was not completely inhibited but only reduced by 60.1% compared to "Ctrl_HR". This is the first time that the effect on STL production was described when *CiGAO* is mutated. The only partial reduction in STL content may suggest that other gene confers the ability to initiate STL synthesis, such as unidentified GAO-like genes or CYP71AV member genes

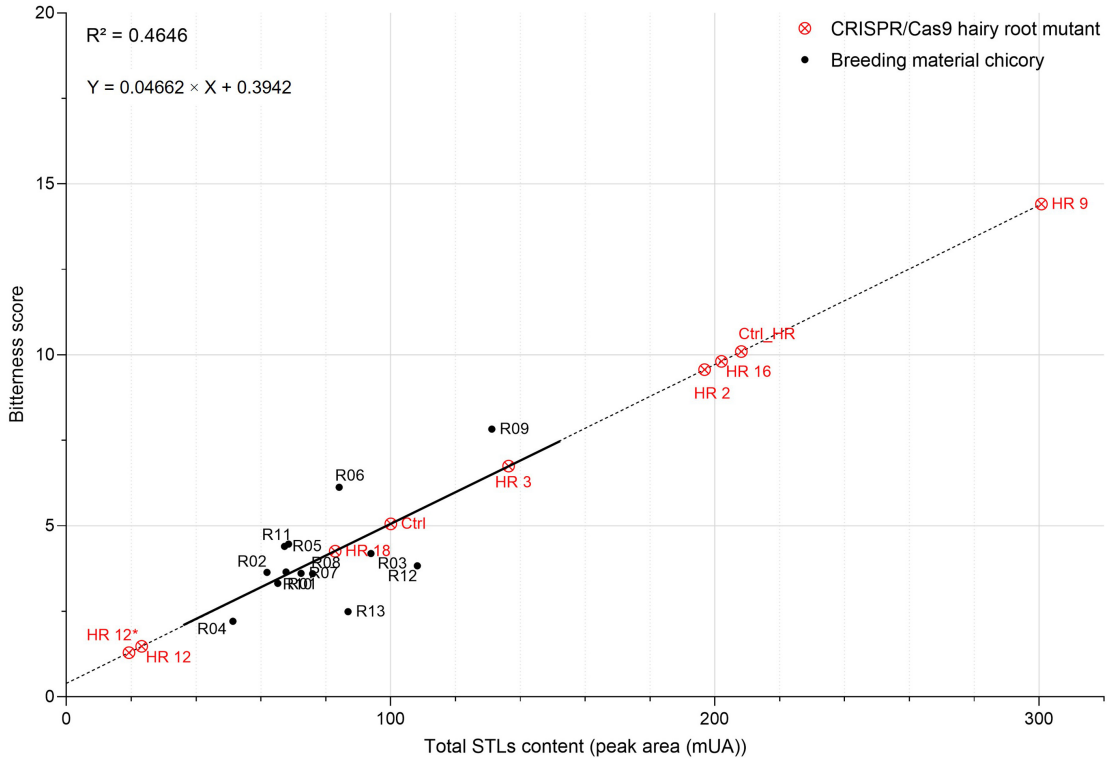

Fig. 6. Relationship between the total STL content and the bitterness score of chicories. Positive correlation (r = 0.68) between these two parameters was established using thirteen field-grown chicories (in black). CRISPR/Cas9 hairy root mutants (in red) were projected onto the linear regression curve using the equation Y = 0.04662 × X + 0.3942, where X was the total STL content of the samples. The significance of these results was confirmed by Pearson's correlation test (two-tailed *p*-value = 0.0103).

that may perform the same function as GAO. In the literature, several expressed copies of *CiGAO* have been identified in *C. endivia* and only one functional gene has been described for *CiGAO* in *C. intybus* [17,46,47]. Given the existence of gene clusters for the *CiGAS-S* and *CiKLS* genes involved in the STL biosynthetic pathway, the hypothesis that multiple active copies of *CiGAO* would exist seems more than likely. However, it should be considered that *CiGAO* is a gene belonging to the large family of cytochromes P450, especially the CYP71AV subfamily [17,48]. It has been shown that members of this subfamily can catalyze the conversion of several sesquiterpenes such as the valencene oxidase (CYP71AV8) which is able to convert germacrene A to its acid *in vitro* [18] or the CYP71AV9, a GAO gene identified in *Cynara cardunculus*, which is capable of partially converting (+)-germacrene D, cascarilladiene and amorpha-4,11-diene to their oxidized products [49]. Since many genes belong to this family, it is possible that some of them take over when *CiGAO* is inhibited. Taking these arguments into account, we can also hypothesize that if the *CiGAS-S1* and *CiGAS-S2* gene mutations have a greater impact on STLs content than the *CiGAO* gene mutation, this may be due to the fact that the *CiGAS* genes are terpene synthases that appear to have greater substrate specificity than cytochrome P450. Therefore, it can be assumed that even if other terpene synthases are present in the genome, they do not appear to compensate for the loss of several copies of *CiGAS-short*, resulting in greater inhibition of STLs synthesis.

In this work, additional information on the players in the STL biosynthesic pathway have also been provided. Bogdanović *et al.* [16] had previously described the genomic organization of the *CiGAS* genes with the identification of four copies of *CiGAS-short* form and a single gene for *CiGAS-long* form. The analysis of the industrial chicory ChicBitter002 genome carried out in this work enabled the identification of an additional copy named *CiGAS-S5*. However, the action of this newly identified gene copy on root STL content could not be confirmed in *C. intybus*. Over the last ten years, it has been shown that genes involved in the same biosynthetic pathway are sometimes colocalized in one region of the genome in several plants [50]. This gene clustering may cover a complete or near-complete biosynthetic pathway, or it may only be partial, involving a cluster of 2 or 3 genes encoding enzymes from 2 or 3 consecutive steps in a biosynthetic pathway [51]. For the biosynthetic pathway of STLs, it has been previously published that *CiGAS-S1*, *CiGAS-S2* and *CiGAS-S3* were mapped on the same linkage group (LG3), confirming an initial localization of one *CiGAS-short* form gene in the genetic reference map for chicory [16,52]. In our case, we found the same data with the first

three copies of *CiGAS-short* form colocalized with *CiGAO* on the same contig (**Supplementary Fig. 3**). Therefore, we physically established the colocalization of 3 active copies of *CiGAS-short* form genes and one active copy of the *CiGAO* gene, two genes involved in the consecutive enzymatic transformation of FPP into germacrene A for the first step and to germacrene A to germacrene A acid for the second step. Moreover, preliminary Blastp alignements of our *CiGAS-S5* sequence to the nr database (Asterid section) under the NCBI site have indicated one candidate sequence (Protein_id=KAI3765470.1) from the *C. intybus* genome of Fan *et al*. [53], which is different from the sequence revealed with 3 *CiCSAS-S1*, *S2* and *S3* as entries (Protein_id=KAI3781194.1). According to the NCBI database informations, both sequences are on separate linkage group localizations, LG3 and LG2 for KAI3765470.1 and KAI3781194.1, respectively. Further, KAI3765470.1 (supposed *CiGAS-S5* locus) is located to 60.1kb of a region with two predicted sequences (KAI3765465.1 and KAI3765466.1) with strong sequence homologies with our *CiGAS-S4b*, including the interruption in the exon 3 (data not shown). Overall, there is some evidence that *CiGAS-S5/CiGAS-S4b*, which were on a separate contig from the 3 *CiGAS-S1* to *CiGAS-S3* copies according to our data, are actually on different chicory chromosomes.

With the goal of using chicory as a functional ingredient, bitterness modulation represents a great importance for manufacturers and STLs are known to contribute predominantly to this bitterness in *C. intybus* [8–10]. In our work, the determination of the STL content was performed using an extraction mimicking food consumption condition and we considered that the total STL content was a good marker to assess bitterness, as confirmed by the correlation established in our sensory analysis. In fact, studies combining sensory analysis and identification of the most bitter compound are not always in agreement: on the one hand, Price *et al*. [8] consider lactucin glycoside to be the most bitter STL, while on the other hand, Van Beek *et al*. [9] states that 11(S),13-dihydrolactucopicrin is the most bitter due to its very low perception threshold. Furthermore, the various studies conducted on the characterization of STLs do not establish that a particular abundance of any form of STLs can influence the perception of bitterness. For example, Ferioli *et al*. [54] reported that glycosylated STLs were the most abundant forms in chicory, accounting for an average of 60% of the total STL content, and Graziani *et al*. [14] claimed that oxalated forms were the least abundant STLs in chicory, with the concentration of 11(S),13-dihydrolactucopicrin-15-oxalate accounting for about 0.2 to 2% of the total content of STLs. These data were contradicted by Kips [55] who reported that oxalate forms were the most abundant STLs in chicory, which was confirmed by Bogdanović *et al*. [29] and Twarogowska *et al*. [56] who found a two-to-four-fold higher oxalate content than the other STLs, apart for 11(S),13-dihydrolactucin and 11(S),13-dihydro-8-deoxylactucin. In the end, there is no consortium on the most bitter compound, but it is accepted that all STLs contribute strongly to bitterness. These inconsistencies may be due to various reasons such as cultivar, growing conditions and techniques, storage conditions and duration, or the method of drying or extraction of the chicory root. In addition, in most papers, chicory root extracts are hydrolyzed or undergo enzymatic treatment to release the bound forms of STL and allow the evaluation of a total content that better correlates with sensory bitterness as the STL content pool is closer to what the consumer perceives when chicory products are consumed [16,46,57,58].

In addition of their involvement in bitterness, STLs play a role in plant defense such as antibacterial or antifungal activities. The lettucenin A, a STL of lettuce, has been shown to have antimicrobial activity against *Bremia lactucae*, *Botrytis cinerae* and *Pseudomonas syringae* phytopathogens [59] and the study of Wedge *et al*. [60] showed the antifungal activity of 6 STLs against *Colletotrichum acutatum*, *Phytophtora fragariae*, *Phomopsis* sp. and *B. cinerae* phytopathogens. To date, no study has tested the antimicrobial activity of chicory root extracts against phytopathogens but only against human pathogenic strains. However the presence of hydroxycinnamic acids, in particular chlorogenic acid and isochlorogenic acid, in large quantities in chicory roots do not allow to conclude the involvement of STLs [61–63]. STLs are also known for their antiparasitic activities [64], and for this purpose, chicory is used as a forage plant. Since these chicories require high levels of STLs, CRISPR/Cas9 could also be used not to reduce bitterness but rather to increase the levels of STLs, perhaps by acting directly on transcription factors.

5. Conclusions

Interest in using *C. intybus* as a functional ingredient has increased in the last few years and chicory flour has recently been shown to have multiple health benefits [5,6]. Many articles have focused on the use of chicory root as a functional ingredient in food products such as yogurts and biscuits but also as chicory flour to be added to crackers, cakes and bread [7,65–70]. However, the amount of chicory used in these products never exceeds 5% because its bitterness causes a rejection by consumers. Several debittering methods have been investigated in chicory over the past years. For example, a change in the growing conditions can be responsible for a less bitter chicory taproot, chicory roots can be bleached or soaked into water for several hours, or low bitter taproot chicories can be bred by classical breeding approach [56,71,72]. However genetic engineering can also be used in chicory to reduce STL content in *Cichorium intybus* L. CRISPR/Cas9 is a useful tool for elucidating gene function that has been used to help breeding and to improve crops such as maize, rice, or wheat [23,73–76]. In this work, we successfully used the CRISPR/Cas9 system to inactivate *CiGAS-short* form

and *CiGAO* genes and showed that biallelic mutations are required to reduce STL content. Given the limitations of current European legislation on the use of CRISPR/Cas9-modified plants, it was not possible to perform a sensory analysis and establish a direct link between bitterness and the amount of STLs in HR chicory lines. Therefore, an indirect link was established by performing sensory analysis of thirteen field-grown chicories, which allowed us to define a bitterness scale based on STLs content and to establish a theoretical bitterness score for the HR mutant lines. We identified three HR mutant lines with significantly reduced STLs content: the HR12 and HR12* lines, that have a theoretical bitterness score between 1 and 2 meaning that bitterness is almost no more perceived, and the HR18 line which has a bitterness score decreased by 57.8% compared to the "Ctrl_HR" which is much more bitter than industrial chicories grown in fields because of the integration of T-DNA in ChicBitter002. Using the CRISPR/Cas9 tool, we were able to modulate the bitterness of *C. intybus* and identify the genes responsible for this. To our knowledge, this is the first time that the effect of CRISPR/Cas9 edited chicory on bitterness can be assessed, even indirectly. The new CRISPR/Cas9-based biotechnology has proven once again to be more efficient and accurate, less energetic and less expensive in time than the other techniques of debittering. As plants obtained by using CRISPR/Cas9 technology are considered to be genetic modified organisms by the current European legislation they cannot be used directly but will provide useful data to breeders to develop and propose less bitter varieties of chicory by molecular marker-assisted breeding. The mutants are also an excellent research material to study the biological activities of STLs.

Special Note

The witloof sequence used in the identification of the sequences in the current article, also called Cargese sequence, was obtained in the framework of the Cargese program (2012–2014). It aimed to obtain an annotated chicory sequence through a partnership of 6 private companies (Rijk Zwaan, Enza Zaden, Bejo, Vilmorin, Hoquet Endives, Florimond Desprez) and the CEA (Genoscope, Commissariat à l'Energie Atomique, Evry, France). The RNA-seq data were obtained as part of the Qualichic program (2012–2016), a result of a collaboration between the University of Lille and the company Florimond Desprez.

Abbreviations

COS, costunolide synthase; FPP, farnesyl-pyrophosphate; GAO, germacrene A oxidase; GAS, germacrene A synthase; HR, hairy root; KLS, kauniolide synthase; KO, knockout; LG, linkage group; sgRNA, single-guide RNA; STL, sesquiterpene lactone; WT, wild-type.

Availability of Data and Materials

The genome of chicory ChicBitter002 used during the current study are not publicly available due to their belonging to private consortium. The protein sequences of GAS-S and GAO analyzed during the current study are available under the accession number given in this paper.

Author Contributions

TC, PH and CR designed the research study. JD, MT, AE and HADS performed the research. JD and TC analyzed the genomic data. MT and PH analyzed the sensorial data. JD and PH performed the statistical analysis. JD and CR conceptualized and drafted the manuscript. DG and JLH contribute to the conception of the work, and to the analysis, the interpretation of data for the work. JD, TC, PH, DG, and CR reviewed and edited the manuscript. CR supervised the project. JLH and CR provided fundings and administrated the project. All authors contributed to editorial changes in the manuscript. All authors read and approved the final manuscript.

Ethics Approval and Consent to Participate

The study was conducted in accordance with the Declaration of Helsinki and informed consent was obtained from all subjects involved in the study.

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Conflict of Interest

The authors declare no conflict of interest.

Supplementary Material

Supplementary material associated with this article can be found, in the online version, at https://doi.org/10.31083/j.fbl2809201.

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