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**DOI**

[10.1080/21645698.2019.1681868](https://doi.org/10.1080/21645698.2019.1681868)

**Publication date**

2020

**Document Version**

Final published version

**Published in**

GM crops & food

**License**

Article 25fa Dutch Copyright Act

[Link to publication](#)

**Citation for published version (APA):**

Bogdanović, M., Cankar, K., Dragičević, M., Bouwmeester, H., Beekwilder, J., Simonović, A., & Todorović, S. (2020). Silencing of germacrene A synthase genes reduces guaianolide oxalate content in *Cichorium intybus* L. *GM crops & food*, 11(1), 54-66.  
<https://doi.org/10.1080/21645698.2019.1681868>

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RESEARCH PAPER



## Silencing of germacrene A synthase genes reduces guaianolide oxalate content in *Cichorium intybus* L.

Milica Bogdanović <sup>a</sup>, Katarina Cankar <sup>b</sup>, Milan Dragičević <sup>a</sup>, Harro Bouwmeester <sup>c</sup>, Jules Beekwilder <sup>b</sup>, Ana Simonović <sup>a</sup>, and Slađana Todorović <sup>a</sup>

<sup>a</sup>Institute for Biological Research “Siniša Stanković”- National Institute of Republic of Serbia, University of Belgrade, Belgrade, Republic of Serbia; <sup>b</sup>Wageningen Plant Research, Wageningen, The Netherlands; <sup>c</sup>Plant Hormone Biology group, Swammerdam Institute for Life Sciences, University of Amsterdam, Amsterdam, The Netherlands

### ABSTRACT

Chicory (*Cichorium intybus* L.) is a medicinal and industrial plant from the Asteraceae family that produces a variety of sesquiterpene lactones (STLs), most importantly bitter guaianolides: lactucin, lactucopicrin and 8-deoxylactucin as well as their modified forms such as oxalates. These compounds have medicinal properties; however, they also hamper the extraction of inulin – a very important food industry product from chicory roots. The first step in guaianolide biosynthesis is catalyzed by germacrene A synthase (GAS) which in chicory exists in two isoforms – GAS long (encoded by *CiGASl*) and GAS short (encoded by *CiGASsh*). AmiRNA silencing was used to obtain plants with reduced GAS gene expression and level of downstream metabolites, guaianolide-15-oxalates, as the major STLs in chicory. This approach could be beneficial for engineering new chicory varieties with varying STL content, and especially varieties with reduced bitter compounds more suitable for inulin production.

### ARTICLE HISTORY

Received 14 May 2019  
Revised 14 October 2019  
Accepted 15 October 2019

### KEYWORDS

*Cichorium intybus*;  
sesquiterpene lactones;  
guaianolides; germacrene  
A synthase; gene silencing;  
amiRNA

### Introduction


Chicory (*Cichorium intybus* L.) is a widely used medicinal and industrial plant from the *Asteraceae* family. Numerous chicory varieties are cultivated for different purposes. The upper parts of the plant are used as salad and vegetable (chicons), both slightly bitter. The underground taproot is an important source of inulin, a fructose polymer commonly used in the food industry. Its traditional uses include animal feed, a cheap coffee replacement, and many medicinal applications.<sup>1</sup> Lately, chicory is viewed by the industry as functional food – nutritious with benefits for human health, and its importance is increasing, especially in developing countries, due to relative ease of cultivation and low agricultural input.<sup>2</sup>

Chicory's pest resistance, as well as its bitter flavor, is due to the presence of secondary metabolites, namely sesquiterpene lactones (STLs), accumulating largely in the latex of the plant.<sup>3</sup> STLs are responsible for some of the medicinal properties of chicory, including antifungal,<sup>4</sup>

antimalarial,<sup>5</sup> anti-inflammatory, anti-tumor,<sup>6</sup> and cytotoxic activity.<sup>7</sup> However, they can also act as allergens and irritants.<sup>8</sup> Bitter compounds in chicory are of economic importance as well. Since some varieties are used for human consumption, and consumers prefer different bitterness levels depending on the market and traditional uses,<sup>9</sup> having a wider selection of less bitter varieties could push chicory use to new markets. Chicory roots are also a major source of inulin, used in the food industry as prebiotic, sweetener, fat-replacing and texturizing agent. The extraction of inulin is hindered by the co-extraction of STLs,<sup>10</sup> raising the cost of inulin production. Thus, having varieties with lowered STL production would be highly beneficial.

The most diversified and abundant group of STLs in chicory are the guaianolides,<sup>11</sup> all derived from a common precursor – germacrene A.<sup>12</sup> Present both in leaves and roots of the plant, the most common guaianolides in chicory are lactucin, lactucopicrin, and 8-deoxylactucin, as well as

**CONTACT** Milica Bogdanović  [milica.bogdanovic@ibiss.bg.ac.rs](mailto:milica.bogdanovic@ibiss.bg.ac.rs)  Institute for Biological Research “Siniša Stanković”- National Institute of Republic of Serbia, University of Belgrade, Bul. despota Stefana 142, 11000 Belgrade, Republic of Serbia

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their modified forms – oxalates, sulfates and glycosides, with oxalates being the most abundant form.<sup>3</sup> These compounds are mainly responsible for the bitter taste of chicory and lettuce.<sup>6</sup> The first step in guaianolide biosynthesis is the conversion of farnesyl diphosphate (FPP) to germacrene A (Figure 1), catalyzed by germacrene A synthase (GAS).<sup>12</sup> This enzyme is encoded in chicory by two genes, whose cDNAs – *CiGASlo* (long isoform) and *CiGASsh* (short isoform) – have been isolated previously.<sup>19</sup> The two isozymes have 72% similarity at the amino acid level, and both convert FPP to germacrene A, as confirmed by heterologous expression.<sup>19</sup> *CiGASlo* transcript is encoded by a single gene mapped on a locus on LG9, and *CiGASsh* is mapped on one locus on LG3 with at least three gene copies, two the result of a recent duplication.<sup>13</sup> However, not much is known about the function of the two isoforms *in planta*. Elucidating the function of these genes would be beneficial for the production of new chicory varieties with different STL content, which has been slow using traditional methods since the species is self-incompatible with high heterozygosity level.<sup>20</sup>

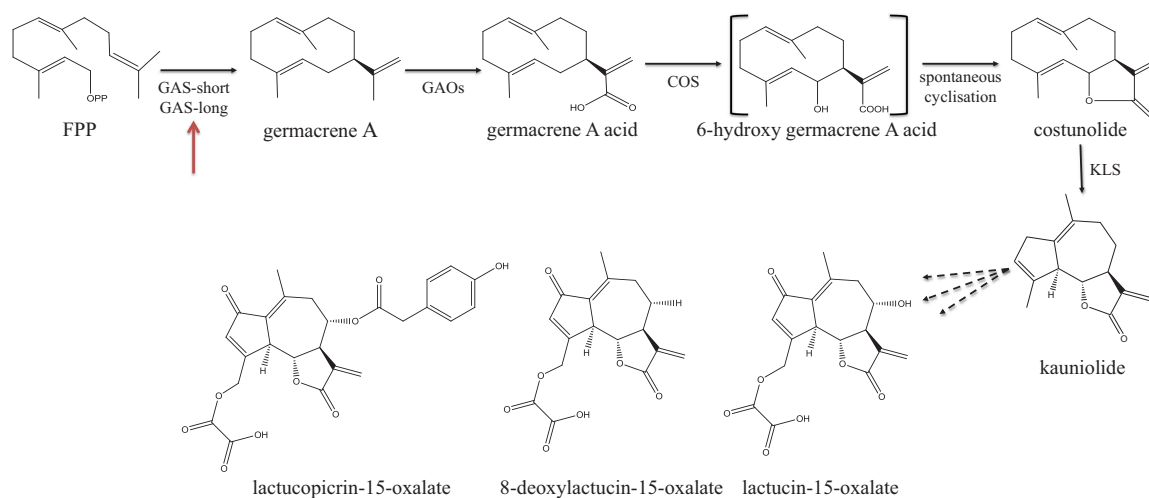
Gene silencing is one of the methods used to study gene function in plants and bioengineer plant varieties. Unlike the conventional approach by producing mutant lines, RNA interference (RNAi) offers a quicker and cheaper alternative to obtain mutant phenotype with benefits of high specificity, dominant phenotype regardless of gene

copy number and production of lines with varying degree of silencing.<sup>21</sup> RNAi has the potential in plant metabolic engineering to increase or decrease the production of the desired compounds<sup>22</sup> or to suppress their degradation.<sup>23</sup> In this study, RNAi was used to produce chicory lines with decreased production of STLs using amiRNA approach, which was shown to be more specific and efficient than using longer hairpin constructs.<sup>21,24,25</sup>

## Material and Methods

### Vector Construction

Silencing constructs were designed according to *CiGASlo* (GenBank: AF497999) and *CiGASsh* (GenBank: AF498000) mRNA sequences. The cloning procedure is given in Supplementary figure 1. AmiRNAs were amplified from pRS300 vector (Addgene, USA) by replacing a sequence of Arabidopsis micro RNA (miR319a) with a desired sequence through a series of overlapping PCR reactions.<sup>26</sup> The reactions were performed with Phusion High-Fidelity polymerase (New England Biolabs Inc., USA) according to the manufacturer's protocol (Supplementary tables 1 and 2). The mixture contained 200 ng pRS300 DNA and 0.4  $\mu$ M primers from Supplementary table 3. Primers were designed according to Schwab et al.<sup>26</sup> AmiRNA sequences specific for either *CiGASlo* (amiGASlo, TATCTAAGAT-



**Figure 1.** STL biosynthesis in chicory and the most abundant guaianolide compounds, modified from Bogdanović et al.<sup>13</sup> GAS – germacrene A synthase,<sup>12</sup> GAOs – germacrene A oxidases,<sup>14,15</sup> COS – costunolide synthase,<sup>16,17</sup> KLS – kauniolide synthase.<sup>18</sup> Silencing target – GAS is marked by the red arrow. Dashed lines mark several consecutive steps in biosynthesis.

ATCTTCACCGT) or *CiGASsh* gene (amiGASsh, TA-ATAGTTTGTCAAGCTGCGC), were chosen based on good hybridization with the target ( $-35$  to  $-40$  kcal mol<sup>-1</sup>) and very low off-target binding. Amplified amiRNAs were first cloned into pDONR207 (Invitrogen, USA) using Gateway® BP Clonase® Enzyme Mix (Invitrogen, USA) and recombinant colonies were selected on LB plates supplemented with gentamicin. The presence of the insert was confirmed by colony PCR and the cloned fragment was sequenced using DYEnamic ET Terminator Cycle Sequencing Kit (GE Healthcare, UK) with primers given in Supplementary table 3. Selected clones were transferred by three-way Gateway reaction into pKGW-RR-MGW for expression in plants using Gateway® LR Clonase® II Enzyme mix (Invitrogen, USA), under the control of 35S promoter and terminator. The vector also contains a streptomycin/spectinomycin bacterial resistance gene, a kanamycin plant resistance gene and DsRED as a fluorescent marker for transformation under the control of AtUBQ10 promoter.<sup>27</sup> Successful cloning of amiRNA fragment was confirmed by colony PCR for the insert and vector-insert borders (Supplementary table 3). The plasmids were named pKGW-amiGASlo and pKGW-amiGASsh.

### Establishment of Chicory *in Vitro* Culture and Regeneration of Transformed Plants

*Cichorium intybus* L. Blue (Samen Mauser, Switzerland) was used for all experiments, and its *in vitro* culture was maintained as described earlier.<sup>28</sup> *Agrobacterium rhizogenes* A4M70GUS contained pRIA4 plasmid with integrated GUS cassette in the T<sub>L</sub> region.<sup>29</sup> Expression vectors were inserted by electroporation. Transformation with A4M70GUS strains carrying amiRNA constructs was conducted as described before for the empty A4M70GUS strain.<sup>28</sup> Regenerated shoots forming spontaneously on root cultures were excised and grown separately.

Genomic DNA of suspected transformants was extracted from leaves of 1-month-old plantlets using a mini-prep CTAB method<sup>30</sup> and treated with RNase A (Fermentas, USA) using the manufacturer's protocol. The PCR mixtures consisted of 100 ng of genomic DNA, 1 μM specific primers (Supplementary table 3) and standard components

according to Fermentas protocol for Taq recombinant polymerase, in a 25 μl volume. The primers specific for DsRED were used to confirm the presence of pKGW-amiRNA plasmids, while virD1-specific primers were used to exclude bacterial contamination. Transgenic plants originating from different HR clones were grown as rosettes for 10 weeks before being used for gene expression and guaianolide oxalate content analyses. Clone lines were propagated as hairy-root cultures. Stable transgene expression in clone lines was further confirmed by observing DsRED fluorescence detected macroscopically using a green LED light (515–530 nm) with a red long-pass 600 nm emission filter, and by confocal microscopy using Leica TCS SP5 II with 543 nm excitation line, 500–530 nm detection and a HCX PL APO CS 20.0 × 0.70 DRY UV objective.

### Quantification of GAS Gene Expression by qRT-PCR

RNA was isolated from chicory roots and shoots using the CTAB method.<sup>31</sup> Total RNA was treated with DNase I (Fermentas, USA) according to the manufacturer's protocol and reverse transcribed using RevertAid First Strand cDNA Synthesis Kit (Fermentas, USA). Quantification of *CiGASlo* and *CiGASsh* gene expression in untransformed controls (eight genotypes of parent plants used for transformation) and plants expressing pKGW-amiGASlo and pKGW-amiGASsh silencing constructs (19 clones originating from these eight genotypes) was performed by qRT-PCR. The reactions were set with Maxima SYBR Green mix (Fermentas, USA), with cDNA corresponding to 100 ng RNA and 0.3 μM primers (Supplementary table 3). The amplification and the preparation of standards for absolute quantification were carried out as described previously.<sup>28</sup> Constitutive expression of 18S rRNA gene was confirmed in parallel using universal plant 18S rRNA primers.<sup>32</sup> qPCR results were analyzed using 7000 System SDS Software.

### Quantification of Guaianolide Oxalates by HPLC-HESI-MS/MS

Sesquiterpene lactone oxalate (lactucin-15-oxalate, 8-deoxylactucin-15-oxalate and lactucopicrin-15-oxalate) content was quantified in untransformed

plants and plants expressing pKGW-amiGASlo and pKGW-amiGASsh silencing constructs. The same material was used for qRT-PCR and metabolite analyses.

Chicory root and shoot (100 mg and 400 mg, respectively) were frozen and powdered in liquid nitrogen. Extraction was performed using 0.133% formic acid in methanol. Samples were sonicated for 10 min and then centrifuged for 5 min at 21000 g at room temperature. Extraction was repeated twice, and the two supernatants were combined and diluted with deionized water in a 1:1 ratio. The extracts were then filtered with Minisart® RC15 (Sartorius, Germany) filters.

Chicory extracts were analyzed with a Dionex Ultimate 3000 UPLC system (Thermo Fisher Scientific, Bremen, Germany) coupled to a TSQ Quantum Access MAX triple-quadrupole mass spectrometer with HESI source (Thermo Fisher Scientific). The chromatographic separation was achieved using a 50 × 2.1 mm Hypersil gold C18 column (Thermo Fisher Scientific), with 1.9 µm particle diameter. Mobile phases were: A – 0.1% Formic acid and B – acetonitrile. The elution gradient was as follows: 0–3 min 5–20% B; 3–5 min 20–40% B; 5–7.5 min 40–50% B, 7.5–8.5 min 50–60% B; 8.5–10 min 60–95% B, 10–13.5 min 95% B, 13.5–14 min 95–5% B, followed by re-equilibration till 18 min with 5% B at a constant flow of 0.4 ml min<sup>-1</sup> and temperature of 30 °C. The vaporizer temperature was set to 100 °C, voltage –3500 V, capillary temperature 275 °C, sheath gas pressure 30 AU, ion sweep gas pressure 0 AU, auxiliary gas flow 7 AU, capillary offset –35 V, tube lens offset –65 V. For qualitative analyses full scan, product ion scan, and parent ion scan were utilized, while for quantification multiple reaction monitoring (MRM) was used. For MRM detection the following mass transitions were used: 8-deoxylactucin-15-oxalate m/z 331 [M-H]<sup>-</sup> → m/z 259, collision energy (CE) 10 eV and m/z 331 → m/z 215, CE 15 eV; lactucin-15-oxalate m/z 347 [M-H]<sup>-</sup> → m/z 275, CE 15 eV and m/z 347 → m/z 213, CE 15 eV, lactucopicrine-15-oxalate m/z 481 [M-H]<sup>-</sup> → m/z 242, CE 15 eV and m/z 481 → m/z 213, CE 20 eV.<sup>3,33</sup> For all fragmentation experiments, the collision gas pressure was held at 1.5 mTorr. Due to lack of authentic standards, relative

quantification was performed by normalizing the peak intensity of each analyte in each sample to the maximal measured peak intensity of the corresponding analyte.

### Data Analysis

Statistical analysis of silencing effect on GAS gene expression and guaianolide oxalate content was performed using R Software<sup>34</sup> and MASS package for R.<sup>35</sup> Comparison of organ dependent *CiGASlo* and *CiGASsh* expression as well as guaianolide oxalates in control plants was evaluated using Wilcoxon signed-rank test. The effects of transformation with silencing constructs on the reduction of *CiGASlo* and *CiGASsh* expression were statistically analyzed using Factorial Nested ANOVA, using the type of construct (control, pKGW-amiGASlo, and pKGW-amiGASsh) and plant part (shoot and root) as the two tested factors. Expression data in different plant parts were nested within the corresponding clones (three samples per plant part per clone). To estimate the effect of transformation on downstream metabolite content, the relative compound quantity of 8-deoxylactucin-15-oxalate, lactucin-15-oxalate, and lactucopicrin-15-oxalate was compared in the three groups: control, amiGASlo, and amiGASsh, independently in roots and shoots using one way ANOVA. Box-Cox power transformation was used to stabilize the dependent variable variances (in order to remove heteroscedasticity) prior to ANOVA, and normality and homoscedasticity of transformed data were confirmed by Levene's test and by checking the residual and quantile-quantile plots. Statistically significant differences were estimated using Tukey's *post hoc* test. The effect of *CiGASlo* and *CiGASsh* expression (continuous explanatory variable) on the relative content of three guaianolide oxalates (response variable) in shoots and roots was tested using multiple linear regression.

## Results and Discussion

### Chicory Transformation with Silencing Constructs

Chicory was transformed with silencing constructs designed to be specific for either *CiGASlo* (pKGW-amiGASlo) or *CiGASsh* (pKGW-amiGASsh), using

*A. rhizogenes* rather than *A. tumefaciens*.<sup>28</sup> The advantage of *A. rhizogenes* transformation is that no plant hormones are required; hairy root (HR) morphology facilitated the selection and regeneration of transformed plants, which occurred spontaneously and rapidly. Initial selection of root clones was performed based on the HR phenotype (Figure 2(a)), rather than kanamycin resistance (due to *nptII*, present on the binary vector), since kanamycin is less efficient in co-transformation events, as it does not discriminate against chimeric roots.<sup>36</sup> Selected root clones spontaneously regenerated shoots (Figure 2(b)) which were grown separately and displayed normal plant morphology (apart from better developed and branched roots), as expected from previous results.<sup>28,37,38</sup> The obtained chicory plants were tested by genomic PCR (Figure 2(c)) to select transgenic plants carrying the amiRNA constructs. T-DNA from the silencing vectors was present in 118 out of 183 tested plants (64.48%) (Table 1). A similar co-transformation efficiency was achieved using the MSU440 *A. rhizogenes* strain in *Lotus japonicus*.<sup>39</sup> Interestingly, not all regenerated chicory plants originating from the same HR clone were transformed, suggesting the chimeric nature of hairy-roots as reported by Limpens et al.<sup>36</sup> Roots of most confirmed plants were reliably expressing the DsRED marker over several months in culture (Figure 2(d,e)), with variable intensity. Only two transgenic plants that did carry a silencing construct had no observable fluorescence in the roots at any time point, while four plants had transient expression present only in the first subculture. Transgene expression from binary plasmids can vary depending on

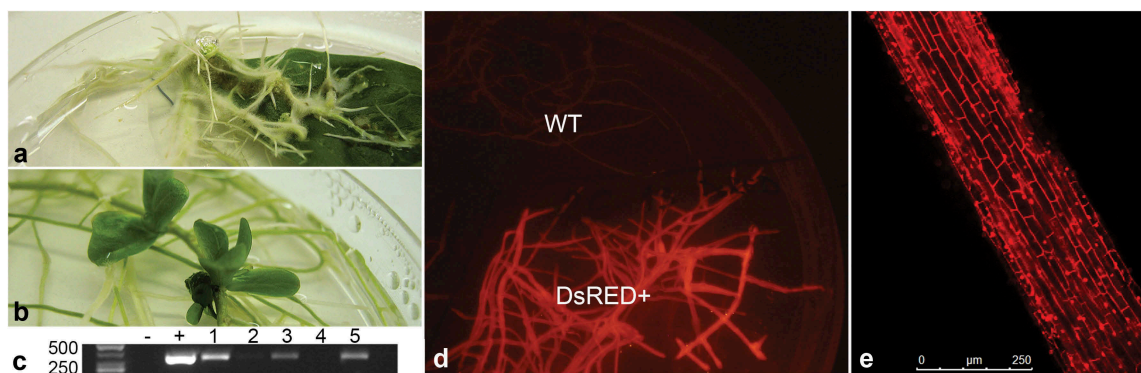
integration site, gene silencing, and other factors,<sup>40</sup> and is usually present in 20–50% of HR clones obtained from *A. rhizogenes*-transformed plants.<sup>36,41</sup>

### GAS Gene Expression Is Reduced in Transformed Clones

QRT-PCR analysis revealed that both *CiGASlo* and *CiGASsh* were expressed in *in vitro* regenerated untransformed plantlets, with *CiGASsh* transcripts being on average 12.3 times more abundant in roots and 6.24 times more abundant in shoots in comparison to *CiGASlo* (Figure 3). In a previous study, Bouwmeester et al.<sup>19</sup> showed, using semi-quantitative Northern analysis, that *CiGASlo* had higher expression in all tested tissues than *CiGASsh*, except in taproot inner tissue, where *CiGASsh* was predominantly expressed, and in whole green seedlings where the expression of the two genes was comparable. Similar results

**Table 1.** Summary of chicory transformation efficiency using *A. rhizogenes* strains carrying silencing constructs. Plants were tested by PCR for the integration of the DsRED gene.

Construct	pKGW-amiGASlo	pKGW-amiGASsh
Initial number of HR clones	90	64
Number of HR clones that regenerated plants	37	37
Number of HR clones tested positive for DsRED	15	20
Number of regenerated plants tested for DsRED	86	97
Number of regenerated plants tested positive for DsRED	46	72



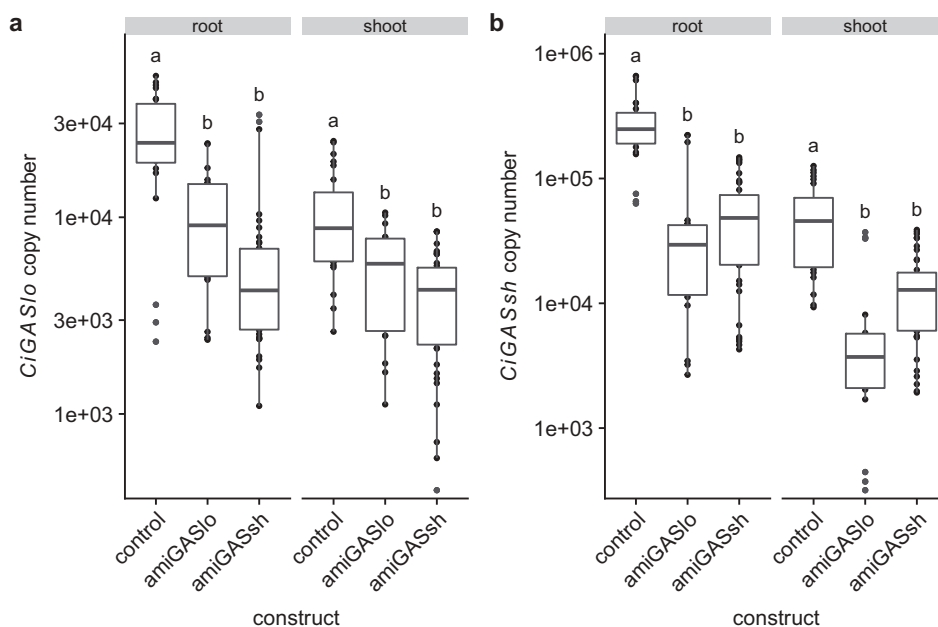
**Figure 2.** Chicory transformation with *A. rhizogenes* strains carrying silencing constructs. (a) Hairy-roots developing on inoculated leaves 17 days after transformation. (b) Spontaneously regenerated shoots of one HR clone. (c) DsRED gene presence or absence in genomic DNA of tested plants 1–5 (amplicon length: 349 bp), bacterial vector DNA (+), no template control (-). (d) DsRED fluorescence of one transformed HR clone (DsRED+), compared to non-transformed root (WT). (e) Confocal micrograph of DsRED fluorescence in epidermal root cells of an HR clone.

were obtained by our group using plants grown in the greenhouse.<sup>13</sup> It is well known that the gene expression commonly differs between *in vitro* and *ex vitro* cultured plants.<sup>42</sup> Promoter activity analysis using promoter-eGFP fusions confirmed that the two isoforms had different promoter activity, with the *CiGASlo* promoter being active in both roots and shoots, while the *CiGASsh* promoter was predominantly active in roots.<sup>13</sup>

The expression of *CiGASsh* and *CiGASlo* was significantly higher in roots as compared to shoots for both control and transformed clones, as revealed by ANOVA (with  $p$ -values  $p < .001$  and  $p < .01$ , respectively). This was confirmed for control plants by nonparametric Wilcoxon signed-rank test ( $p < .05$  for both gene expression). These results are in accordance with previous results on chicory from greenhouse obtained by Bouwmeester et al.<sup>19</sup> and Bogdanović et al.<sup>13</sup> In *Lactuca sativa*, where STLs also accumulate in latex, two germacrene A synthases – *LTC1* and *LTC2* – were found to be expressed constitutively in roots, but only *LTC2* was highly expressed in leaves.<sup>43</sup> In species where terpenes accumulate mainly in leaf trichomes, *GAS* genes are more active in young leaves than in roots, as in *Artemisia annua*,<sup>44</sup> *Tanacetum*

*parthenium*,<sup>45</sup> and sunflower.<sup>46</sup> In *Achillea millefolium*, where volatile terpenoids constitute the most important fraction, the highest expression of *GAS* gene was detected in flowers and leaves, and much less in rhizome, root and stem tissues.<sup>47</sup>

In order to reduce the guaianolide production in chicory, two *GAS* genes that convert FPP to germacrene A (Figure 1) were selected as silencing targets. Since amiRNAs, which imitate natural miRNAs, are more specific and more reliable for predicting possible off-target effects as compared to long hpRNA constructs,<sup>24</sup> this approach was chosen for silencing. Comparison of *CiGASlo* and *CiGASsh* expression in control (untransformed) plants and selected pKGW-ami*GASlo* and pKGW-ami*GASsh* transformants (showing DsRED fluorescence) confirmed that silencing was successful (Figure 3). The silencing of two *GAS* genes was significant, with transcript levels reduced up to 30.8 times for *CiGASlo* and up to 70.7 times for *CiGASsh* as compared to parent plants (Table 2). Variable level of silencing in different transgenic lines (Figure 3) is probably due to a positional effect of amiRNA integration<sup>48,49</sup> and can be useful to select lines with adequate silencing levels to help characterize gene function<sup>50</sup> or to produce varieties with differing STL levels. ANOVA



**Figure 3.** *CiGASlo* (a) and *CiGASsh* (b) expression (transcript copy number per cDNA corresponding to 100 ng RNA, determined by absolute quantification) in roots and shoots of controls and plants transformed with pKGW-ami*GASlo* and pKGW-ami*GASsh* constructs. To stabilize the expression data variance  $\log$  transformation was used prior to visualization and statistical analyses. Statistically significant differences within roots or shoots, obtained by Tukey's *post hoc* test at  $p < .05$  are marked with different letters.

analysis indicated that there are no significant differences in the strengths of *CiGASlo* and *CiGASsh* silencing in shoots and roots; in other words, either gene was silenced in both shoots and roots by a similar degree.

Surprisingly, silencing constructs were not specific toward either of the two genes. Since these two genes have a similar coding sequence – 72% identity on the deduced amino acid level<sup>19</sup> and 75% identity over 83% query cover at the mRNA level (Supplementary figure 2), amiRNAs could be directly affecting the nonspecific transcript, a problem usually present when the two transcripts have five or fewer mismatches in amiRNA target region.<sup>26</sup> In our system, amiGASlo has nine mismatches compared to *CiGASsh*, and amiGASsh has six mismatches to *CiGASlo* (Supplementary figure 2), not counting the last nucleotide in the target region, which is always modified to A in amiRNA. Non-target silencing can also be a consequence of feedback regulation, when the two genes participate in the same signal transduction chain,<sup>26</sup> as in the case of MADS box genes in *Arabidopsis*.<sup>25</sup> MiRNAs can also be involved in transitive RNAi – spreading of the silencing outside of the initial target sequence.<sup>51</sup> The proposed mechanism for this action is second-strand synthesis and trans-acting siRNA (small interfering RNA) production, mediated by RNA-dependent RNA polymerases, which in turn target other mRNAs for miRNA-like cleavage.<sup>51</sup> Even though amiRNAs are designed having in mind rule sets to reduce nonspecific targeting and transitive RNAi,<sup>52</sup> amplification of the silencing signal can be dependent on other factors which are harder to control, like structural characteristics of the primary transcript and amiRNA construct.<sup>53,54</sup>

## GAS Gene Silencing Reduces Guaianolide Oxalate Content

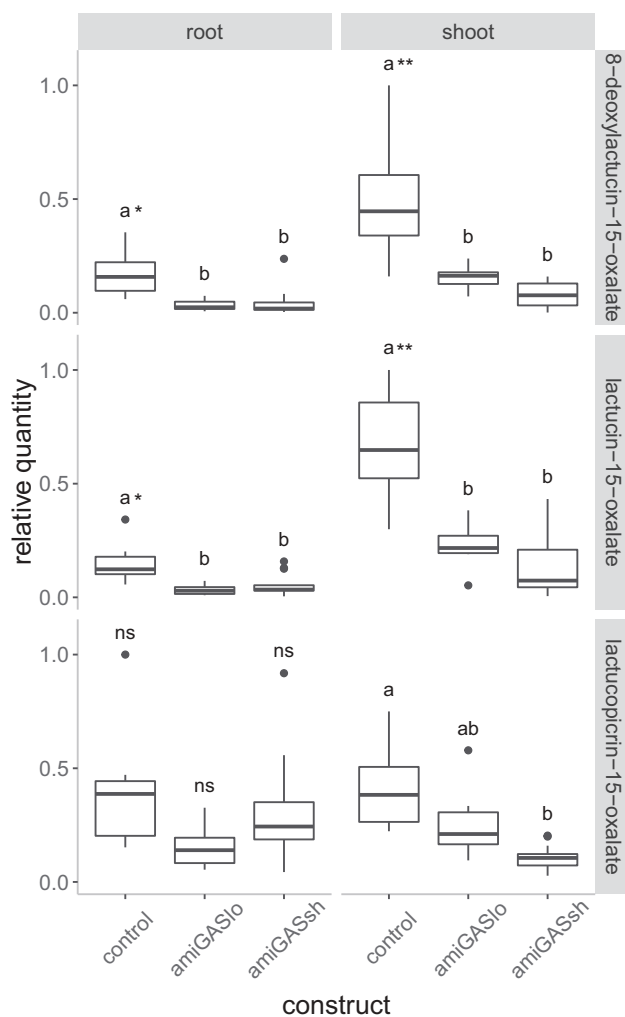
To evaluate the impact of silencing of the GAS genes on guaianolide production, the concentration of three downstream metabolites, 8-deoxylactucin-15-oxalate, lactucin-15-oxalate, and lactucopicrin-15-oxalate was analyzed by HPLC-HESI-QqQ. The three compounds were selected since they were present in high amounts in untransformed plants and could reliably be identified by comparing MS/MS fragmentation patterns (Supplementary figure 3) with literature data.<sup>3,33</sup> Guaianolide oxalates constitute the main fraction of STLs in chicory and are more abundant than free guaianolides,<sup>3</sup> so we expected their quantity to reflect the effects of silencing. Guaianolide oxalates were indeed detected in both roots and shoots of chicory plants (Figure 4). Our findings corroborated the results obtained by Sessa et al.<sup>3</sup> who identified guaianolide-15-oxalates as major STLs in both lettuce and chicory.

The relative abundance of lactucin-15-oxalate and 8-deoxylactucin-15-oxalate was significantly higher in the shoots than in the roots of control plants ( $p < .01$ , Wilcoxon signed-rank test), with a shoot-to-root ratio in individual plants varying from 2.44 to 7.89 for lactucin-15-oxalate and 1.50 to 6.54 for 8-deoxylactucin-15-oxalate. The content of lactucopicrin-15-oxalate did not differ significantly in shoots and roots ( $p < .01$ , Wilcoxon signed-rank test), with shoot-to-root ratio in individual plants varying from 0.48 to 3.64. To our best knowledge, there are no data on organ distribution of guaianolide-15-oxalates in chicory, while data for related species are limited.<sup>3</sup> In chicory at the rosette stage, free guaianolides are usually more abundant

**Table 2.** Fold change of GAS gene expression and guaianolide content in selected transformed clones compared to their corresponding parent plants. Bold values are values with maximum fold reduction.

Construct	Clone	Organ	<i>CiGASlo</i>	<i>CiGASsh</i>	8-deoxy-lactucin-15-oxalate	Lactucin-15-oxalate	Lactucopicrin-15-oxalate
pKGW-amiGASlo	5-1 2	shoot	3.9	27.1	6.2	4.8	2.9
	5-1 2	root	2.7	<b>70.7</b>	45.6	14.8	6.5
	5-15 4	shoot	0.8	5.1	2.9	17.5	0.8
	5-15 4	root	1.0	8.0	18.7	21.8	<b>8.7</b>
pKGW-amiGASsh	26-10 4	shoot	3.6	0.7	<b>166.4</b>	4.2	8.1
	26-10 4	root	3.4	45.3	10.4	2.7	1.0
	26-14 2	shoot	<b>30.8</b>	2.2	4.1	6.1	2.5
	26-14 2	root	5.0	4.5	17.4	12.5	2.5
	27-18 4	shoot	6.3	43.3	19.9	<b>185.2</b>	8.5
	27-18 4	root	13.5	28.0	7.5	7.2	4.8





**Figure 4.** Relative quantity of guaianolide oxalates: 8-deoxylactucin-15-oxalate, lactucin-15-oxalate and lactucopicrin-15-oxalate in roots and shoots of control and pKGW-amiGASlo and pKGW-amiGASsh transformed plants. To stabilize the relative quantity variance Box-Cox power transformation was used prior to statistical analyses ( $\log$  for 8-deoxylactucin-15-oxalate in roots, lactucin-15-oxalate in roots, lactucopicrin-15-oxalate in shoots and roots and square root for 8-deoxylactucin-15-oxalate and lactucin-15-oxalate in shoots). Statistically significant differences obtained by Tukey's *post hoc* test at  $p < .05$  are marked with different letters (ns – no significant differences in ANOVA). Statistically significant differences of lactucin-15-oxalate and 8-deoxylactucin-15-oxalate content in roots and shoots of control plants obtained by Wilcoxon signed-rank test ( $p < .01$ ) are denoted with \* and \*\*.

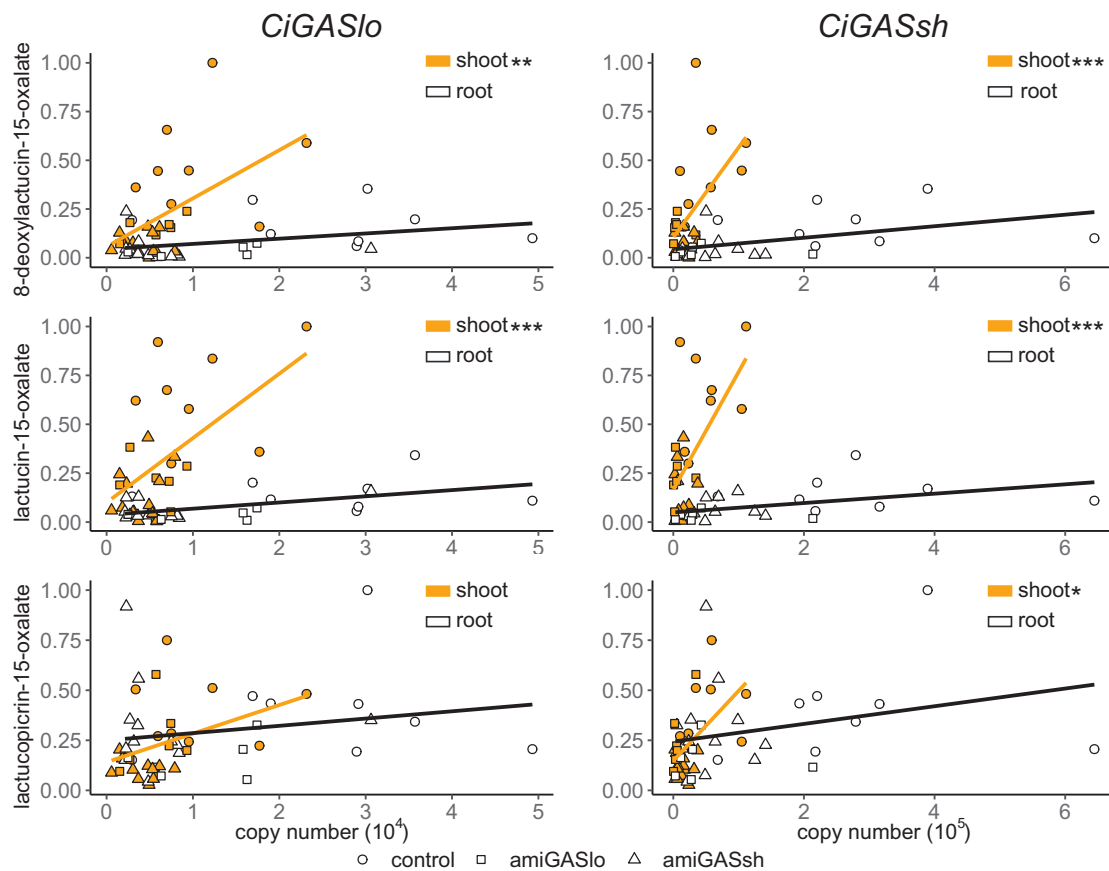
in the root than in the shoot,<sup>13</sup> but their content may change during development<sup>55</sup> and particularly upon flowering.<sup>28,55,56</sup>

Prior to discussing the effects of silencing on guaianolide oxalates content, it should be noted that the transformation with *A. rhizogenes per se*, e.g. its *Rol* genes, can boost the production of

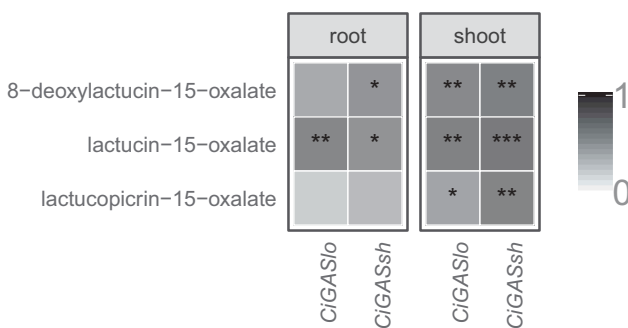
secondary metabolites in a variety of species.<sup>57</sup> However, chicory plants transformed with the same (but empty) *A. rhizogenes* strain, A4M70GUS had more free guaianolides only at the flowering stage, while at the rosette stage (used in the current setup) guaianolide content was comparable in transformed and control plants.<sup>28</sup>

Introduction of the GAS-silencing constructs resulted in a significant reduction of the guaianolide oxalate content (Figure 4): up to 185.2 times for lactucin-15-oxalate, up to 166.4 times for 8-deoxylactucin-15-oxalate and up to 8.7 times for lactucopicrin-15-oxalate in some of the clone lines, as compared to the levels in parent plants (Table 2).

To elucidate whether the levels of guaianolide-15-oxalates in different parental and transgenic clones depend on the level of GAS gene expression, the relationships between relative abundance of guaianolide-15-oxalates and the expression of *CiGASlo* and *CiGASsh* in roots and shoots of tested clones are presented as scatter plots (Figure 5) and as Person's correlation heatmaps (Figure 6). The obtained data suggest that lactucin-15-oxalate and 8-deoxylactucin-15-oxalate accumulate significantly less with decreasing expression of GAS genes in the shoots (Figure 5) whereas in roots this correlation is less clear. Also, the correlation between *CiGASsh* and *CiGASlo* expression and compound content is higher and more significant in shoots (Figure 6). The correlation between *CiGAS* gene expression and guaianolide oxalate content in roots depends on the compound, with lactucin-15-oxalate having the highest significant correlation and lactucopicrin-15-oxalate having no significant correlation (Figure 6). These results suggest that the accumulation of guaianolide oxalates can be altered by manipulation of GAS expression in the shoots, probably reflecting natural means of regulation of their synthesis. On the other hand, regardless of higher *CiGAS* gene expression in roots (Figure 4), it seems that other mechanisms or enzymatic reactions (e.g. any of the steps from germacrene A oxidation to conjugation with oxalic acid, see Figure 1) may control the accumulation of guaianolide oxalates in roots. Also, their (in)stability and turnover may be different in different organs, while their polar nature<sup>3</sup> may provide a higher



**Figure 5.** Scatter plots of the relationship between the relative compound abundance of guaianolide oxalates (8-deoxylactucin-15-oxalate, lactucin-15-oxalate, and lactucopicrin-15-oxalate) and expression (copy number) of *CiGASlo* and *CiGASsh* genes in roots and shoots of clone lines (control plants –  $\circ$ , plants transformed with pKGW-amiGASlo –  $\square$  and with pKGW-amiGASsh –  $\Delta$ ). The statistical significance of the interaction of plant part (root and shoot) and gene expression on compound content was estimated using multiple linear regression,  $p < .05$  is marked with \*,  $p < .01$  is marked with \*\* while  $p < .001$  is marked with \*\*\*.



**Figure 6.** Pearson's correlation heatmap between relative compound abundance of guaianolide oxalates (8-deoxylactucin-15-oxalate, lactucin-15-oxalate, and lactucopicrin-15-oxalate) and expression (copy number) of *CiGASlo* and *CiGASsh* genes in roots and shoots. Significance of correlation is marked with \* for  $p < .05$ , \*\* for  $p < .01$  and \*\*\* for  $p < .001$ .

degree of mobility within the tissues and laticifer network in comparison to nonpolar STLs.

To the best of our knowledge, this is the first successful report on reducing the concentration of bitter guaianolide compounds by gene silencing in plants. There are reports describing silencing effects and reduction of other terpenoid groups, like volatile terpenoids in tobacco<sup>58</sup> and cotton,<sup>59</sup> and monoterpene alkaloids in *Catharanthus roseus*,<sup>60</sup> with gene expression reduced between 4 and 6.67 times, and compounds of interest reduced between 1.43 and 10 times. In a transient silencing assay by agroinfiltration in tobacco, two RNAi constructs were able to silence *5-epi-aristolochene synthase*, an enzyme involved in sesquiterpenoid biosynthesis, 3.4 and 6.2 times compared to the control expression value, with *5-epi-aristolochene* emission consequently reduced 8.4 and 2.8 times compared to the control emission level,

respectively.<sup>61</sup> Silencing of squalene synthase, producing the substrate for triterpene and sterol biosynthesis, was also reported to be 2.7 and 4.2 times reduced compared to control expression levels.<sup>61</sup> The gene for this enzyme was also silenced in apple by virus-induced gene silencing by 1.72 fold.<sup>62</sup> Recently, silencing of amorpho-4,11-diene synthase, which encodes the first step in artemisinin biosynthesis, was achieved in *Artemisia annua*.<sup>63</sup> The authors report a stable *Agrobacterium* transformation of *A. annua* using hpRNA constructs and selecting several lines with substantial gene silencing – 25 times reduction of gene expression compared to untransformed control plants. When assessing the content of artemisinin, a cadinanolide STL (a compound synthesized several steps downstream from the silenced gene, which is comparable to our system), the authors also found a substantial reduction of 20-fold in selected clone lines. However, when measuring amorpho-4,11-diene, a direct product of the silenced gene, no reduction could be observed, but there was a significant increase of this compound in young leaves of silenced lines compared to controls and a comparable level in mature or dry leaves of clones to controls.<sup>63</sup> These results suggest that the effect of silencing genes from the diverse group of terpenoid synthases can be difficult to predict and interpret. In our system, some lines displayed a reduction of gene expression and STL oxalate content of several orders of magnitude (Table 2), suggesting that gene silencing with amiRNAs could be a good method for the production of low-bitterness chicory clones.

## Conclusions

In conclusion, amiRNA constructs proved to be a powerful, though not isoform-specific, tool for silencing of two *GAS* genes in chicory. The silencing success was clearly demonstrated not only at the level of expression of *CiGASlo* and *CiGASsh*, but more importantly at the level of downstream metabolites, guaianolide-15-oxalates, the major STLs in chicory. This approach and the obtained clones may have practical application in

engineering varieties with reduced guaianolide content, facilitating inulin extraction and obtaining products of superior quality.

## Abbreviations

amiRNA	artificial micro RNA
<i>CiGASlo</i>	Chicory germacrene A synthase, long isoform
<i>CiGASsh</i>	Chicory germacrene A synthase, short isoform
GAS	germacrene A synthase
hpRNA	hair-pin RNA
HR	hairy roots
miRNA	micro RNA
MS	Murashige and Skoog basal medium
RNAi	RNA interference
STL	sesquiterpene lactone
WT	wild type

## Acknowledgments

This work was supported by the Ministry of Education, Science and Technological Development of the Republic of Serbia under Grant no. ON173024 and the European Commission EU FP7 Project: TERPMED under Grant agreement no. 227448. We thank Dr Rene Geurts for supplying pKGW-RR-MGW vector, and Milica Simonović for help with a graphic design of Supplementary figure 1.

## Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

## Funding

This work was supported by the European Commission FP7 [227448]; Ministry of Education, Science and Technological Development of the Republic of Serbia [ON173024].

## ORCID

Milica Bogdanović  <http://orcid.org/0000-0001-8034-2606>  
 Katarina Cankar  <http://orcid.org/0000-0001-5149-4192>  
 Milan Dragičević  <http://orcid.org/0000-0002-9422-2952>  
 Harro Bouwmeester  <http://orcid.org/0000-0003-0907-2732>  
 Jules Beekwilder  <http://orcid.org/0000-0003-3238-4427>  
 Ana Simonović  <http://orcid.org/0000-0003-4909-4493>  
 Slađana Todorović  <http://orcid.org/0000-0002-8233-9037>

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