CRISPR/Cas9-mediated Targeted Mutagenesis of Inulin Biosynthesis in Rubber Dandelion

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ABSTRACT. Rubber dandelion (Taraxacum kok-saghyz) is a natural rubber-producing dandelion that has the potential to become an industrial crop. Inulin is a storage carbohydrate in rubber dandelion, and its synthesis competes with rubber production for assimilated carbon. We used the clustered regularly interspaced short palindromic repeats (CRISPR)/CRISPR-associated protein (Cas) system to simultaneously target two sites in the gene that encodes 1-fructan: fructan-1-fructosyl transferase gene (1-FFT), a key enzyme in inulin biosynthesis. Agrobacterium rhizogenes and Agrobacterium tumefaciens-mediated plant transformation methods were used to generate transgenic plants with CRISPR/Cas9 elements. Transformation rates were 71% and 64% via A. rhizogenes and A. tumefaciens-mediated transformations, respectively. Mutagenesis was confirmed by the loss of restriction site method and Sanger sequencing. Of 13 transgenic plants obtained via A. rhizogenes, six showed editing of both target sites within the 1-FFT gene. Transgenic rubber dandelion plants were obtained within 10 weeks using A. rhizogenes-mediated transformation, which was much faster than the 6 months required for A. tumefaciens transformants. Of 11 transgenic plants obtained via A. tumefaciens, five showed mutations in both target sites. Reverse-transcription polymerase chain reaction confirmed Cas9 expression in all edited transformants. Both A. rhizogenes-mediated double-mutant transformants and A. tumefaciens-mediated double mutant transformants had lower levels of inulin than wild-type plants. Moreover, A. rhizogenes-mediated transformants had a higher rubber content than wild-type plants. Therefore, the present study validates the use of CRISPR/Cas9 gene editing as an efficient tool for the generation of useful mutations in rubber dandelion and could be implemented in future crop improvement approaches.
In addition, the CRISPR/Cas9 system can efficiently introduce biallelic or homozygous mutations in transgenic plants that can be stably segregated into the next generations (Brooks et al. 2014; Fauser et al. 2014; Shan et al. 2013; Zhang et al. 2014). However, this technology has not been widely used in industrial crops such as rubber dandelion (*Taraxacum kok-saghyz*) (Iaffaldano et al. 2016).

Rubber dandelion is being developed to produce commercially viable levels of high-quality natural rubber and diversify the rubber supply geographically and biologically. All commercially available natural rubber in the world comes from a single tropical plant, the Para rubber tree (*Hevea brasiliensis*), which is cultivated as genetically identical clones mostly in southeast Asia (Mooibroek and Cornish 2000). These *Hevea* production areas are challenged by many fungal and viral pathogens. In 2019, *Pestalotiopsis* and *Neofusicoccum ribis* leaf drop fungal pathogens spread to $4 \times 10^5$ ha in only 6 months, causing up to 90% natural rubber production loss in some areas of Thailand (International Rubber Consortium 2020). Global natural rubber production decreased 10% ($1.4 \times 10^7$ kg) in 2020 because of these blights, extreme weather events, and labor shortages (Association of Natural Rubber Producing Countries 2020). Furthermore, *Hevea* plantations are faced with restricted land availability in their limited growing range, increasing labor costs, and a deforestation moratorium (Grogan et al. 2019; Van and Poirier 2007). Rubber dandelion is an alternative natural rubber source that can be mechanically cultivated as an annual crop and harvested and processed in geographic areas with temperate climates (Ramirez-Cadavid et al. 2017). However, rubber dandelion is largely undomesticated, and cost-effective field production of rubber is not yet possible because of slow growth rates, poor competition with weeds, and a short growing season (Mooibroek and Cornish 2000). Only very few studies have been performed to increase the agronomic performance of rubber dandelion using genetic engineering approaches (Iaffaldano et al. 2016). CRISPR/Cas9 could be an ideal approach to enhance the quantity of natural rubber in rubber dandelion.

Natural rubber in rubber dandelion is synthesized in root lactifers and stored as a terminal sink of carbon assimilated from CO₂. However, considerably more carbon is stored as inulin, which is the principal storage carbohydrate in this species. Inulin is metabolized to survive winter weather and fuel rapid leaf production during the following spring (Iaffaldano et al. 2016). Suppressing inulin biosynthesis may increase the flux of photosynthetic carbon toward rubber production. A previous postharvest study showed that carbon generated by the release of sugars during inulin catabolism in harvested roots can flow back to the rubber biosynthetic pathway during cold storage in the dark; furthermore, each gram of inulin created ~0.5 g of new rubber (Cornish et al. 2013). Overexpression of the inulin catabolism gene 1-fructan 1-exohydrolase (*I-FFT* gene) in a subset of transgenics nearly doubled the accumulation of cis-1,4-polyisoprene in rubber dandelion and the closely related species *Taraxacum brevicaulicore*um, as determined by °H-NMR, but the molecular weight was not determined (Stolze et al. 2017). In the current work, we applied a CRISPR/Cas9-driven gene editing approach to mutagenize the 1-fructan: fructan-1-fructosyl transferase gene (*I-FFT*) encoding fructan: fructan-1-fructosyl transferase, one of the key enzymes involved in inulin biosynthesis (Van Laere and Van den Ende 2002). The efficiency of the CRISPR/Cas9 system was increased by targeting two sites of the same gene compared with a single target site (Iaffaldano et al. 2016; Xing et al. 2014). With the enhanced mutation efficiencies, mutations should be more efficiently transmitted to the next generation.

**Materials and Methods**

**Plant material.** Seeds of rubber dandelion Foxtrot, a fifth-generation advanced population selected at the Ohio State University, Wooster, OH, USA, from the US Department of Agriculture (USDA) 2008 rubber dandelion collection, were used for CRISPR/Cas9-induced mutagenesis. Seeds were surface-sterilized with 15% bleach for 20 min, followed by rinsing five times with autoclaved water and then germinated on solid half-strength Murashige and Skoog (1/2 MS) medium with micronutrients and macronutrients (Caisson Laboratories, Inc., North Logan, UT, USA) supplemented with Gamborg’s B5 vitamins, 20 g L⁻¹ sucrose, and 2 g L⁻¹ Gelrite (Sigma-Aldrich, St. Louis, MO, USA) (Gamborg et al. 1968; Murashige and Skoog 1962). The plants were maintained at 23 to 27°C under a 16-h/8-h light/dark photoperiod (Zhang et al. 2015).

**Selection of target sequences and CRISPR/Cas9 construct design.** The CRISPR/Cas9 target sequences were selected within the second exon of *I-FFT* gene because it is present in all predicted isoforms (Iaffaldano et al. 2016). Potential CRISPR sites within this exon were identified using CRISPRdirect (Naito et al. 2015). Then, these sequences were manually entered into NEBuilder version 2.0 (Vinzce et al. 2003) to identify target sites that would result in the loss of a restriction site in the *I-FFT* gene. The target specificities of the selected sequences were evaluated by performing a BLAST against the rubber dandelion draft whole genomes (Lin et al. 2018) and RNA-seq data from six discrete rubber dandelion genotypes (Luo et al. 2017). A CRISPR/Cas9 binary vector with two guide RNA (gRNA) expression cassettes targeting two adjacent sites of the *I-FFT* gene was successfully generated as described previously (Xing et al. 2014) (Supplemental Fig. 1). Briefly, the two target sites were incorporated into polymerase chain reaction (PCR) forward and reverse primers (Supplemental Table 1). For the assembly of two gRNA expression cassettes, a PCR fragment containing the two gRNAs was amplified from the pCBC-DT1T2 plasmid (plasmid #50590; Addgene, Watertown, MA, USA) with two forward primers and two reverse primers that were partially overlapping. This gRNA expression cassette (T1T2-PCR) was purified using a PCR purification kit (Qiagen; Germantown, MD, USA). Then, the fragment was ligated into the binary vector (pKSE401; plasmid #62202; Addgene) using *BsaI* restriction site and T4 ligase (New England Biolabs Inc., Ipswich, MA, USA) in a Golden Gate cloning reaction as described by (Xing et al. 2014) to generate the *I-FFT* CRISPR/Cas9 constructs. The correct orientation of the insert was confirmed with Sanger sequencing and the final construct was introduced into competent *Agrobacterium rhizogenes* K599 and *Agrobacterium tumefaciens* EHA105 by electroporating the cells in a 1-mm gap elec- troproporation cuvette at 25 μF capacitance, 400 Ohms resistance, and 1.8 kV voltage.

**Agrobacterium-mediated plant transformation.** Root and leaf fragments from aseptically-grown rubber dandelion plants were used for *A. rhizogenes* and *A. tumefaciens*-mediated trans- formation, respectively. Root fragments (length, 1–2 cm) from 12-week-old rubber dandelion plants were transformed with *A. rhizogenes* harboring the *I-FFT* CRISPR/Cas9 two-site construct using methods described previously (Zhang et al. 2015).
Hairy roots produced were transferred into solid 1/2 MS medium supplemented with 400 mg L\(^{-1}\) ticarcillin disodium and clavulanate potassium (Timentin; PhytoTech Laboratories, St. Lenexa, KS, USA) and 10 mg L\(^{-1}\) kanamycin. Regenerated plantlets with the hairy root phenotype were used to validate mutation events. Rubber dandelion root fragments were also inoculated with \( \textit{A. rhizogenes} \) K599 wild-type using the same method. These root fragments were regenerated on 1/2 MS medium supplemented with 400 mg L\(^{-1}\) ticarcillin disodium and clavulanate potassium.

Leaves from 6- to 10-week-old rubber dandelion plants were used for \( \textit{A. tumefaciens} \)-mediated transformation. Approximately 1-cm\(^2\) leaf discs were inoculated with \( \textit{A. tumefaciens} \) harboring the \( 1\text{-FF} \) CRISPR/Cas9 two-site construct for 15 min on a shaker at 100 rpm. The leaf discs were blotted dry and cocultured on solid shoot induction medium (SIM) (1X MS, MS micro-salts and macro-salts, Gamborg’s B5 vitamins, 2% sucrose, 0.7% plant tissue culture agar, Phytoagar, Dublin, OH, USA), 0.2 mg L\(^{-1}\) indole-3-acetic acid, and 1 mg L\(^{-1}\) 6-Benzylaminopurine supplemented with 200 μM acetylsyringone. After a 3-d coculture period, the discs were washed with liquid SIM supplemented with 400 mg L\(^{-1}\) ticarcillin disodium and clavulanate potassium and then transferred to solid SIM supplemented with 400 mg L\(^{-1}\) ticarcillin disodium and clavulanate potassium following the protocols described previously (Zhang et al. 2015). After 1 week of recovery, rubber dandelion leaf discs were transferred to SIM plates supplemented with 400 mg L\(^{-1}\) ticarcillin disodium and clavulanate potassium and 5 mg L\(^{-1}\) kanamycin. After 4 weeks, regenerated calli were transferred to solid shoot elongation medium (1X MS, MS micro-salts and macro-salts, Gamborg’s B5 vitamins, 2% sucrose, 0.7% plant tissue culture agar, 0.1 mg L\(^{-1}\) indole-3-acetic acid and 0.5 mg L\(^{-1}\) kinetin) supplemented with 400 mg L\(^{-1}\) ticarcillin disodium and clavulanate potassium and 10 mg L\(^{-1}\) kanamycin. After 4 weeks of shoot elongation, the generated shoots were transferred to root induction medium (0.5X MS, MS micro-salts and macro-salts, Gamborg’s B5 vitamins, 2% sucrose, 0.7% plant tissue culture agar and 0.2 mg L\(^{-1}\) indole-3-acetic acid) supplemented with 400 mg L\(^{-1}\) ticarcillin disodium and clavulanate potassium and 10 mg L\(^{-1}\) kanamycin. Regenerated plantlets were used to validate mutation events. Rubber dandelion leaf discs were also inoculated with wild-type \( \textit{A. tumefaciens} \) EHA105 using the same method.

**Analysis of CRISPR/Cas9-induced genome editing.** Genomic DNA was extracted from leaves of rubber dandelion plants transformed with K599 and EHA105 harboring the 1-FFT CRISPR/Cas9 construct using a CTAB-based extraction method coupled with purification on a silica matrix (Vilanova et al. 2020). DNA from nontransformed wild-type Foxtrot plants was extracted as the negative control. Genomic regions flanking the two targeted genome editing sites were amplified in a 25-μL reaction containing 1X Standard Taq Reaction buffer, 200 μM dNTPs, 0.2 μM forward and 0.2 μM reverse primers, 0.4 U Taq DNA polymerase, and 10 ng genomic DNA. PCR reaction conditions were 2 min of initial denaturation at 95 °C, 30 s of denaturation at 95 °C, 30 s annealing at 65 °C, 60 s of elongation at 72 °C for 30 cycles, followed by a final extension at 72 °C for 5 min. The PCR products were gel-purified using a gel extraction kit (Qiagen). The purified DNA was tested for the presence of mutations by the restriction enzyme site loss method (Parry et al. 1990). The restriction enzymes \( \textit{MluCI} \) and \( \textit{BsrI} \) were used to detect the loss of the cut sites in the first and second target sites, respectively. PCR products from wild type-plants are expected to be digested completely by \( \textit{MluCI} \) and \( \textit{BsrI} \), resulting in two gel bands with sizes of 54 bp and 107 bp for \( \textit{MluCI} \) and 51 bp and 110 bp for \( \textit{BsrI} \). PCR products that cannot be digested completely indicate that the genome is edited, causing the loss of these restriction enzyme sites. Digestion-resistant PCR products were cloned into a plasmid vector using a cloning kit (TORPO\textsuperscript{TM} TA Cloning\textsuperscript{TM} Kit; Thermo Fisher Scientific Inc., Waltham, MA, USA) and analyzed by Sanger sequencing.

Total RNA was extracted from leaf tissues of plants transformed with K599 and EHA105 harboring the 1-FFT CRISPR/Cas9 construct as well as leaves of nontransgenic wild-type Foxtrot plants as a negative control, following the method described by Chomczynski and Sacchi (2006). RNA from each sample was treated with DNase I to remove DNA (TURBO DNA-free\textsuperscript{TM} Kit, Invitrogen\textsuperscript{TM}; Carlsbad, CA, USA). cDNA synthesis was performed using reverse-transcriptase (Superscript\textsuperscript{TM} II; Invitrogen\textsuperscript{TM}). Gene expression analysis for Cas9 was performed using reverse-transcription PCR with 50 ng of cDNA using the aforementioned reaction and procedures. Endogenous gene β-actin was amplified as a control using the same amount of cDNA. All primers used are listed in Supplemental Table 1. PCR products (a total volume of 10 μL) were assessed on 2% agarose gels (weight/volume) stained with ethidium bromide.

Validated transformed plants were transferred into sterile pet pellets soaked in liquid 1/2 MS medium with 400 mg L\(^{-1}\) ticarcillin disodium and clavulanate potassium. After 3 weeks, transformed plants in peat pellets were transferred into pots filled with peat-based soilless media (Pro-Mix; Premier Tech Horticuture, Pointe-Lebel, QC, Canada) and then moved into a greenhouse with a 12-h/12-h (light/dark) photoperiod at 22 °C.

**Quantification of rubber and inulin.** Root samples from plants confirmed to contain a mutated 1-FFT gene were analyzed to determine their inulin and rubber contents. Roots from 3-month-old rubber dandelion plants were harvested and dried as previously described (Lankitus et al. 2023). Briefly, plants were removed from their tree pots, and excess dirt from the roots was shaken loose. Leaves were clipped off the plant slightly above the crown to prevent latex leakage from the crown or the roots. Roots were then weighed, placed in brown paper bags, and dried for at least 2 weeks in a 50 °C oven before being ground to a powder with an analytical grinding mill (Basic analytical mill IKA A10; Millipore Sigma, Billerica, MA, USA). The ground rubber dandelion roots were extracted using an accelerated solvent extraction system (Dionex ASE 200, Thermo-Fisher Scientific) with two 40-min water extractions at 95 °C, followed by one 40-min acetone extraction at 40 °C and three, 20-min hexane extractions at 160 °C. Inulin content in the water extract was determined by ion-exclusion high-performance liquid chromatography (Ramirez-Cadavid et al. 2017). A near-infrared spectroradiometer (FieldSpec\textsuperscript{TM} 3 Spectroradiometer; Analytical Spectral Devices Inc., Boulder, CO, USA) and a previously constructed computer model (Ramirez-Cadavid et al. 2018) were used to determine the rubber content of root hexane extract. Mutated plants with high rubber and low inulin concentrations were interbred to generate seeds for later research.

**Statistical analysis.** A one-way analysis of variance (ANOVA) in a completely randomized design (n = 3–4) was used to analyze root dry weight, inulin, and rubber content data using the general linear model procedure in statistical software (SAS version 9.4; SAS Institute Inc., Cary, NC, USA). Model residuals were tested for normality using residual and predicted plots.
and the Shapiro Wilks test within Proc Univariate. The assumptions of the ANOVA were met, and no transformations were used. Post hoc mean separation among treatments was obtained using the PDIFF option of the LSMEANS commands that made pairwise comparisons of all treatments.

**Results**

**Agrobacterium-mediated transformation in rubber dandelion.** The transformation rate was calculated for *A. tumefaciens* and *A. rhizogenes*-mediated transformation of rubber dandelion by dividing the number of explants that produced callus or shoots by the number of starting explants. Of 70 leaf explants transformed with *A. tumefaciens*, 45 produced calli, resulting in a transformation efficiency of 64.2%; however, of 80 root explants transformed with *A. rhizogenes*, 57 produced calli, resulting in a 71.2% transformation efficiency. Furthermore, transgenic rubber dandelion plants were obtained within 10 weeks using *A. rhizogenes*-mediated transformation of rubber dandelion roots, which was much faster than the 6 months required for *A. tumefaciens*-mediated transformation of leaves. Plants inoculated with both wild-type *A. rhizogenes* and *A. rhizogenes* harboring the CRISPR/Cas9 construct had dense plagiotropic root growth, whereas the roots of uninoculated plants had normal gravitropic growth. The seedlings with a mutated 1-FFT gene had more leaf biomass than wild-type plants and plants inoculated with either wild-type Agrobacterium (Fig. 1).

**Mutation analysis of regenerated plants.** A separate analysis of 20 T0 transgenic lines from *A. rhizogenes*- and *A. tumefaciens*-mediated transformations by restriction enzyme digestion of a PCR fragment spanning the two target sites revealed that 13 of the *A. rhizogenes* transgenic lines had digest-resistant bands for at least one target site and six for both target sites (Fig. 2), whereas 11 of the *A. tumefaciens* transformants had digest-resistant bands for at least one target site and five for both target sites (Fig. 3). Therefore, the approximate mutagenesis efficiencies were 30% and 25% for both target sites in *A. rhizogenes*- and *A. tumefaciens*-mediated transformations, respectively.

We cloned and sequenced the digestion-resistant PCR fragments and found that the sequences between the two target sites had single nucleotide insertions, deletions up to 18 nucleotides, as well as single nucleotide substitutions in CRISPR/Cas9-edited plants (Figs. 2 and 3). These indels resulted in frameshift mutations and truncated and nonfunctional proteins. For *A. rhizogenes*-mediated transformations, there were six transgenic plants with mutations in both sites. Of these, five were heterozygous and one was monoallelic for the first target site and three were heterozygous and three were monoallelic for the second target site. For *A. tumefaciens*-mediated transformants, five plants had mutations in both target sites. Of these, three were heterozygous and two were monoallelic for both target site.

**Cas9 expression in mutated plants.** Reverse-transcription PCR was performed to test the transcripts of Cas9 in the independent transgenic plants that showed mutations in both target sites and showed that Cas9 was expressed in all plants (Fig. 4) (three examples each of *A. rhizogenes-* and *A. tumefaciens*-mediated transformants).

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**Fig. 1.** Agrobacterium rhizogenes- and Agrobacterium tumefaciens-mediated transformation in rubber dandelion. Representative phenotypes of a (A) rubber dandelion wild-type (WT) plant regenerated from a root fragment, (B) transgenic rubber dandelion plant transformed with WT *A. rhizogenes* K599, (C) transgenic plant transformed with *A. rhizogenes* harboring the clustered regularly interspaced short palindromic repeats (CRISPR)/CRISPR-associated protein (Cas) 9 expression cassette, (D) WT plant regenerated from a leaf disc, (E) transgenic plant transformed with WT *A. tumefaciens* EHA105, and (F) transgenic plant transformed with *A. tumefaciens* harboring the CRISPR/Cas9 expression cassette. Size bars indicate 1 cm.
QUANTIFICATION OF RUBBER AND INULIN. The root dry weight was not significantly different among EHA105-FFT, K599-FFT, and wild-type plants ($P = 0.696$) (Fig. 5). However, plants had different phenotypes; EHA105-FFT and wild-type plants had a tap root with thick lateral roots, whereas K599-FFT plants had thin hairy roots. The root inulin concentration in K599-FFT plants was significantly lower than that in wild-type plants ($P = 0.027$), indicating successful mutagenesis of the $1$-FFT gene and, hence, the disruption of the inulin biosynthesis pathway. However, the root inulin concentration in EHA105-FFT plants was not significantly different from that of wild-type plants. Furthermore, the residual (solid) rubber concentration was significantly higher in K599-FFT plants than in wild-type plants ($P = 0.011$), whereas neither concentration was significantly different in EHA105-FFT plants.

Discussion

Developing efficient bioengineering and transformation methods is of great importance to the successful commercialization of rubber dandelion as an industrial crop. Both the $A. rhizogenes$ and $A. tumefaciens$ methods used in this study to generate transgenic plants have advantages and disadvantages.

$Agrobacterium rhizogenes$ has a root-inducing (Ri) plasmid. The T-DNA region of this Ri plasmid has root locus ($rol$) genes, and $rol$ genes will be frequently co-transformed into the plant.
genome along with the gene of interest. Upon expression, these rol genes will alter endogenous plant hormone concentrations and facilitate the production of hairy roots at the site of inoculation in many dicots (Limpens et al. 2004). Transgenic plants can be obtained by regenerating these hairy roots (Christey and Braun 2004; Kuluev et al. 2016) and used as a rapid tool for gene expression studies.

In rubber dandelion, *A. rhizogenes*-mediated transformation is frequently performed using root fragments as explants. Rubber dandelion has the ability to naturally regenerate shoots from root explants on nonhormonal medium in tissue culture (Zhang et al. 2015) and, hence, roots have shown the highest regeneration efficiency in rubber dandelion (Bae et al. 2005; Bowes 1970; Lee et al. 2004). *Agrobacterium rhizogenes* has higher transformation efficiency in *Taraxacum* spp. than *A. tumefaciens* (Bae et al. 2005; Lankitus et al. 2023; Lee et al. 2004). In *Taraxacum platycarpum* leaf discs, the transformation efficiency using *A. tumefaciens* was 1% to 5% (Bae et al. 2005), whereas the transformation efficiency in root fragments using *A. rhizogenes* was 76.5% (Lee et al. 2004).

Despite *A. rhizogenes* having many advantages, the hairy root phenotype produced is unsuitable for a production system. The rol genes have to be segregated out by backcrossing transgenic plants to wild-type plants and then interbreeding heterozygous progeny to regenerate homozygous mutants. Then, these knockouts can be fully characterized without the confounding influence of rol genes. This process can be time-consuming and laborious, and some effort has been made to distinguish transgene efforts in the presence of rol genes to reduce the number of PCR reactions needed to confirm the presence of transgenes (Lankitus et al. 2023).

Unlike *A. rhizogenes*, *A. tumefaciens* virulence genes are disarmed. *Agrobacterium tumefaciens* has been used to transform *Taraxacum mongolianum, T. platycarpum*, and *T. brevicoronica-latum* (Bae et al. 2005; Post et al. 2012; Song et al. 1991). Many studies have used leaf discs as the explant for *A. tumefaciens* transformation (Collins-Silva et al. 2012; Wahler et al. 2009). Unlike root explants, regeneration of transgenic plants from leaf explants involves multiple steps such as callus, shoot induction, and root induction (Collins-Silva et al. 2012; Post et al. 2012). In the present study, rooting of the regenerated shoots in *A. tumefaciens*-mediated transformation required a considerable time, thus delaying the regeneration process. This was also observed for transgenic *T. platycarpum* (Bae et al. 2005) and *Medicago truncatula* (Crane et al. 2006). Although rubber dandelion may only require 67 to 81 d to generate whole plants through *A. tumefaciens*-mediated transformation (Collins-Silva et al. 2012), in the present study, 168 d were required to produce fully regenerated plants, whereas *A. rhizogenes* transformants required only 70 d.

A mutation efficiency of 39.4% to 88.9% was previously reported using *A. Rhizogenes-mediated* transformation (Iaffaldano...
et al. 2016), similar to 30% efficiency during our study. The lower efficiency of 30% double mutant generation using A. rhizogenes is to be expected because the inefficiencies are doubled. All the small indels introduced by CRISPR/Cas9 caused changes in the reading frame, leading to loss-of-function mutations in the alleles. The absence of mutations in some of the transformed plants could be caused by reduced expression of the gRNA and Cas9 transgenes caused by the T-DNA insertion point or T-DNA rearrangement (Ma et al. 2015; Pan et al. 2016).

Inulin, a poly-fructose with a glucose end group, is the main carbon storage carbohydrate in dandelion roots (Schütz et al. 2006; Van Laere and Van den Ende 2002). The inulin metabolic pathway includes three major enzymes, 1-su-crease:su-crease fruc- tosyltransferase (1-SST), 1-FFT, and 1-FEH. 1-SST initiates inu-elin biosynthesis by transferring a fructose moiety from su-rose to the C-1 of a fructose in another sucrose molecule, yielding the trisaccharide 1-kestose in an irreversible reaction. Subsequently, 1-FFT transfers fructose moieties from 1-kestose to sucrose or other fructans, polymerizing inulin. Preferences of 1-FFT for do-nor and acceptor substrates might differ between plant species, resulting in different patterns of inulin polymers (Van Laere and Van den Ende 2002). The 1-FEH enzyme is involved in inulin degrada-tion (Karimi et al. 2021; Stolze et al. 2017; Van Laere and Van den Ende 2002).

In wild-grown and cultivated rubber dandelion, natural rubber concentration increases in late autumn and early winter as well as during winter dormancy, when inulin reserves begin to de-grade (Stolze et al. 2017; Ullmann 1951). Inulin is synthesized in parenchyma cells localized adjacent to the laticifers and the ex-cess carbon generated by the degradation of inulin can be used to synthesize isopentenyl pyrophosphate, the basic building block of natural rubber (Van Laere and Van den Ende 2002). Therefore, metabolic engineering of the inulin biosynthetic path-way to inhibit inulin biosynthesis could redirect excess carbon to laticifers to synthesize natural rubber, as has been seen in post-harvest storage of harvested roots (Cornish et al. 2013).

Interestingly, the K599-FFT plants had significantly lower levels of inulin and higher levels of root rubber than wild-type plants. However, the root inulin concentration in EHA105-FFT plants, although apparently lower, was not significantly different from that of wild-type plants. In addition, the total rubber concentration in EHA105-FFT plants was similar to that in wild-type plants. A preliminary analysis of the genome assembly of the inbred line OH 1004 suggested that T. kok-saghyz has one functional copy (in agreement with Lin et al. 2018) and up to three nonfunctional copies of 1-FFT, whereas it may have up to three functional copies of 1-FEH and two of 1-SST in contrast to the seven poorly expressed 1-FEH genes reported (Lin et al. 2018). The lack of a convincing inulin reduction or rubber in-crease in the EHA105-FFT plants implies that the leaf transfor-mation method did not lead to full expression in the roots, but the underlying reason is unclear. Perhaps the different disruptions to the endogenous plant growth regulators caused by A. rhizogenes and A. tumafaciens had different downstream effects on inulin and rubber biosynthesis. We were not able to detect any evidence indicating that the EHA105-FFT plants were chimeras. Although the rubber extracted from these plants was not character-ized by size exclusion chromatography, all samples appeared physically similar to each other, suggesting that no radical changes in molecular weight occurred.

It is well known that root locus (rol) genes transferred to the plant genome with the gene(s) of interest in A. rhizogenes-mediated plant transformation alter endogenous plant hormone concentrations and the production of secondary metabolites (Bulgakov et al. 2011). Characterization of phenotypic changes induced by wild-type A. rhizogenes K599 strain (without accompanying trans-genes or edited genes) found no rol-induced changes in the root rubber concentration (Lankitus et al. 2023). Thus, the increased rubber levels detected in K599-FFT plants are solely caused by the redirection of excess carbon from the inhibited inulin pathway.

Interestingly, 1-SST has shown 1-FFT activity (Van den Ende et al. 1996). Upon depletion of sucrose, the synthesis of some oligo-fructans by the purified 1-SST at higher enzyme concentra-tions was demonstrated (Van den Ende et al. 1996). The “fructan synthesizing activity” of 1-SST could result in the increased inulin levels observed in the 1-FFT mutant plants. However, under high sucrose concentrations, small fructans can be synthesized by yeast invertase enzymes (Ca irns and Ashton 1993). Moreover, 1-FFTs from plants have shown a high degree of homology to vacuolar invertases from other related and unre-lated plants. Therefore, it is possible that certain invertases in the plant are synthesizing some amount of fructans in mutant plants. The efficiency of 1-FFT knockdown can vary among the different lines, and this can be analyzed with quantitative reverse-transcription PCR. Silencing of the 1-FFT gene was not confirmed at the mRNA and protein levels during the present study.

Latex of Taraxacum species contains abundant quantities of pentacyclic triterpenes, such as lupeol and amyrins, and other molecules that are largely restricted to the genus Taraxacum, such as taraxasterol (Akashi et al. 1994). Excess carbon from inu-lin biosynthesis can also be directed to the synthesis of these triterpenes. In addition to the pentacyclic triterpenes, carbon can also be used for the synthesis of sterols such as cholesterol, sitosterol, and stigmasterol (Post et al. 2012). Excess carbon also may have been diverted to leaf growth because the EHA105-FFT plants seemed to have more leaf biomass (Fig. 1), although the biomass was not quantified in these plants.

CRISPR/Cas9 gene editing could be used to further enhance rubber yield in rubber dandelion. Combining 1-FFT inhibition with overexpression of 1-FEH, which increased the accumula-tion of cis-1,4, polyisoprene (Stolze et al. 2017) in dandelion roots, might significantly enhance rubber accumulation. In addition, simultaneous mutagenesis of multiple genes is a rapid way to study metabolic pathways that involve multiple enzymes.

The introduced CRISPR/Cas9 DNA usually becomes integrated into plant chromosomes and may have a variable impact on the growth and development of edited plants. Depending on the specific characteristics of the cassette, it could potentially disrupt the normal regulation of gene expression and affect plant growth rates, resulting in altered plant size or development patterns. The presence of the CRISPR cassette raises the possibility of off-target effects. These off-target effects could potentially lead to unexpected plant morphology or development changes. In addition, the presence of transgenes in plants can have implications for regulatory considerations, public acceptance, and potential ecological effects if the plants are intended for release into the environment.

The feasibility of segregating out the CRISPR/Cas9 cassette may vary depending on the plant species, the specific target gene, and the transformation and editing methods used. The efficiency of cassette removal can also be influenced by factors such
as the selection pressure applied during plant regeneration and the stability of the introduced modifications. However, in general, it is feasible to remove the CRISPR/Cas9 cassette from edited plants through various strategies. One approach is to use transient expression of the CRISPR/Cas9 components, where the Cas9 protein and guide RNA are introduced into the plant cells, but the CRISPR/Cas9 genes are not integrated into the genome of the plant. This results in the targeted DNA modification without permanent integration of the CRISPR/Cas9 cassette. Zhang et al. (2016) delivered in vitro transcripts of Cas9 and sgRNAs into immature wheat embryos by particle bombardment and generated DNA-free edited wheat. Another strategy involves using gene-editing techniques that use homology-directed repair (Vu et al. 2020). During homology-directed repair-based editing, the donor DNA template with the desired sequence and CRISPR/Cas9 components is introduced. This allows for precise modification of the target gene by using the plant’s own DNA repair machinery. The donor template can be designed to include sequences that facilitate subsequent removal of the CRISPR/Cas9 cassette, such as loxP or other site-specific recombination sites (Vu et al. 2020). Additionally, the development of base editors and prime editors, which enable precise modifications without creating double-strand breaks, may also facilitate the generation of edited plants without the need for the CRISPR/Cas9 cassette integration (Hua et al. 2022). These technologies offer potential ways to achieve targeted modifications while minimizing the presence of foreign DNA sequences in rubber dandelion.

**Conclusion**

The results of this study verified the feasibility of the CRISPR/Cas9 toolkit in the successful editing of the 1-FFT gene in rubber dandelion resulting in metabolic and physiological changes. This is an affordable, efficient, and time-saving platform for genome editing in rubber dandelion, which may raise the root rubber concentration to commercially viable levels of at least 250 kg of extracted rubber per hectare. Because genome editing tools such as CRISPR/Cas9 are widely used in crop improvement, the feasibility of segregating out the CRISPR/Cas9 cassette must be evaluated to minimize the presence of foreign DNA sequences in edited plants.

**References Cited**


