CRISPR/Cas9 Directed Mutation of *Taraxacum kok-saghyz* for Herbicide Resistance

Kandice Pettaway[1], Kyle Benzle[2], Katrina Cornish[3]

[1]Department of Horticulture and Crop Science, The Ohio State University Graduate School, Wooster, OH 44691

ABSTRACT

Although commonly considered a weed, certain species of dandelion (*Taraxacum*) offer a novel source of high quality natural rubber in relatively high concentrations in their roots. *Taraxacum kok-saghyz* cv. Buckeye Gold (TK, BG) in particular is a high yielding variety that is currently under selection for domestication traits and higher rubber yield. In order for this new crop to be economically competitive with rubber tree grown mainly in Southeast Asia, TK will need to be become more amenable to large-scale field production by introducing herbicide resistance. TK (derived from USDA TK-17) was genetically engineered to allow for herbicide resistance to ALS-inhibiting herbicides. This research will greatly expedite the domestication and industrial development of TK natural rubber. Furthermore, the successful production of an herbicide resistant TK will provide a new and profitable commodity crop option for temperate climates and decreased dependency on foreign nations for this strategic natural resource.

INTRODUCTION

Natural rubber is an essential commodity that remains in high demand by many industries and governments requiring its use in countless applications. Products such as tires, medical devices, construction materials and more are dependent on high quality natural rubber. *Taraxacum kok-saghyz* (TK or Buckeye Gold) is being developed to help supplement *Hevea brasiliensis* (the Brazilian rubber tree) rubber, which is currently the main source of this natural product. TK is an herbaceous yellow flowering plant native to Kazakhstan which produces a high quality natural rubber in its roots, similar to that of *Hevea*.

TK is not yet fully domesticated and the production chain, from seed to processing, is still being developed. Because weed control is the most challenging barrier to the domestication of TK, the specific goal of this work has been to produce a non-transgenic herbicide resistant line of this crop using the CRISPR/Cas9 system. We have tested the hypothesis that TK plants can become herbicide resistant using CRISPR/Cas9 to mutate the native *ALS (acetolactate synthase)* TK gene and selecting for transformed/resistant plants. CRISPR/Cas9 is a powerful genome-editing tool that cuts at a specific DNA site using guide RNA. Full integration of TK natural rubber into the industrial supply chain is dependent on producing a TK line more amenable to producers through the use of herbicide resistant genes.

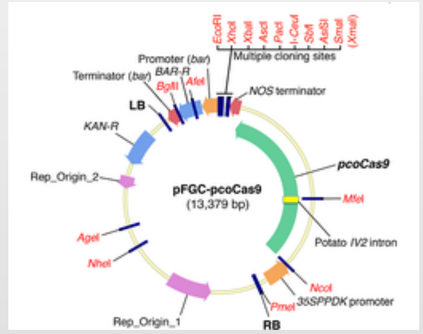
MATERIALS AND METHODS

Plant Material

- We used TK seeds from USDA accession KAZ08-017 (W6 35172).
- 70% ethanol was used to surface sterilize seeds for 1 min, followed by soaking them in 6% sodium hypochlorite for 20 min.
- We rinsed seeds with autoclaved water 3 times and germinated them on solid half strength Murashige and Skoog (½ MS) medium.
- The plants were maintained at 23-27 °C under 16-h light/8-h dark photoperiod with a light intensity of 30 µmol/m^2/s using cool-white fluorescent tubes.

- After 4-6 weeks of growth on ½ MS, we selected the healthiest plants and sub-cultured on TK callus induction media.
- For callus induction, leaf explants about 1.5 cm long were excised with a scalpel and cultured in Petri dishes containing 25 ml ½ MS basal medium supplemented with 10 g/L sucrose, 16 g/L agar and Zeatin and BA at level of ~1 mg/L.
- TK callus induction medium was selected by testing calli production of TK leaves, roots and crowns on induction media containing different combinations of hormones
  - Indole-3-Acetic acid (IAA) at levels 0.1, 0.25, and 0.5 mg/L
  - 2,4-Dichlorophenoxyacetic acid (2,4-D) at levels 0.5, 1.0, 2.0 and 3.0 mg/L
  - Zeatin at levels 0.5, 1.0, 2.0 and 3.0 mg/L
  - 6-Benzylaminopurine (BA) at levels 0.5, 1, and 3 mg/L
- Two leaf explants were cultured in each Petri dish then sealed with plastic wrap and incubated at 25ºC in above conditions and sub cultured every three weeks.
- We used the most green, friable, and clumped callus TK tissue for *Agrobacterium rhizogenes*-mediated transformation

Figure 1.



The full construct (~13 kb) to be used for TK ALS modification is based on the pFGC vector, this carries modified gRNA along with the selection marker.

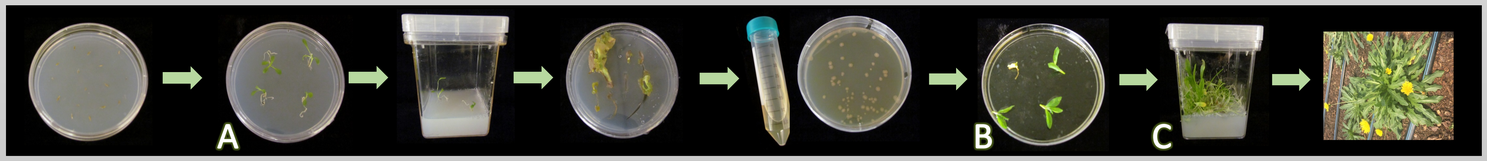
Plasmid

- This Plasmid pFGC-pcoCas9 expresses the Cas9 enzyme (Addgene plasmid # 52256).
- The sgRNA from plasmid pICH86966::AtU6p::sgRNA_PDS was modified to specifically target sites W560L, A639I, P183S/H and G640 in the ALS gene using Q5® Site-Directed Mutagenesis Kit (New England Biolabs Inc., Ipswich, MA, USA).
- These primers were used to amplify the plasmid pFGC-pcoCas9 to replace the original sgRNA targeting sequence with the given sgRNA sequence.
- The constructs were named pAT_W560, pAT_A639,pAT_P183S/H, and pAT_G640.
- We introduced these constructs into *A. rhizogenes* K599 wild type by the heat shock method, and used for plant transformation.

Plant transformation

- Calli and roots were removed and sectioned from the surface of the plantlets.
- The slightly wounded plant tissue were dipped into *A. rhizogenes* cell suspensions for about 10 seconds (Zhang et al., 2015).
- We then placed the plant tissue on ½ MS medium with 200 µM acetosyringone.
- After 3-days of co-culture and a 7-day recovery period (Zhang et al., 2015), the calli and roots were transferred to ½ MS medium with 400 mg/L Timentin.

Figure 2.

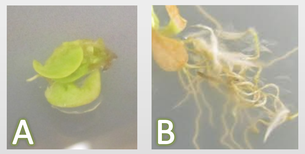


Agrobacterium-mediated transformation using hydroponically grown TK roots, calli, and plantlets as target tissue. (A) TK plants are grown in ½ MS liquid media in sterile conditions, (B) plants are dissected and roots and crowns are used as target tissue for transformation, (C) spectinomycin is used as a selection agent and hairy roots form.

RESULTS

- Calli induced by optimized hormone treatment (Zeatin and BA at levels of ~1 mg/L) produced small yet friable tissue clumps.
- Out of five target sites that we would have liked to modify, we were successful in building three separate constructs with the targeting gRNA.
- Three rounds of TK transformation were completed with the three constructs.
- Construct pAT_P183 and pAT_A639 have produced hairy-root phenotype plants and are now under selection.

Figure 3.



(A) Callus tissue dipped in the agrobacterium containing the construct pAT_P183 produced a single hairy root. (B) A plantlet dipped in the agrobacterium containing the construct pAT_A639 produced multiple white, fuzzy hairy roots.

CONCLUSION/FUTURE WORK

- Callus was able to produce a hairy root, however, growing the callus tissue adds additional time to the project. After a couple of weeks, we could compare the the time it took each type of plant tissue to grow hairy roots. We could also compare the length and number of hairy roots grown on each type of plant tissue. This information would prove or disprove the need for calli in my project.
- If plants are transformed and grow multiple hairy roots, seeds will be germinated from the transformed plants and grown in the field to test TK's resistance to ALS inhibiting herbicides.

BIBLIOGRAPHY

1. Cornish, K. (2001). Biochemistry of natural rubber, a vital raw material, emphasizing biosynthetic rate, molecular weight and compartmentalization, in evolutionarily divergent plant species. Nat. Prod. Rep. 18, 182–189.

2. Zhang, Y., Iaffaldano, B., Xie, W., et al. Rapid and hormone-free *Agrobacterium rhizogenes*-mediated transformation in rubber producing dandelions *Taraxacum kok-saghyz* and *T. brevicorniculatum*, Industrial Crops and Products, 66, 4, 2015.

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