Observable but extremely infrequent off-target mutations in CRISPR/Cas9 expressing transgenic Populus and Eucalyptus trees engineered for containment

Greg S Goralogia[1], Isabella M Andreatta[1], Kelly J Vining[1], Qin Xiong[1,2], and Steven H Strauss[1*]

[1] *Oregon State University, Corvallis, USA*

[2] *Nanjing University, Nanjing, China*

[*] Steve.Strauss@OregonState.Edu

As a containment technology, we generated presumptively sterile *agamous* and *leafy* loss-of-function poplar trees that constitutively express CRISPR/Cas9. Because of the difficulty of removal of CRISPR components in trees and other clonal crops, it is important to understand the potential for off-target mutation that may occur over several years.

We used a targeted sequencing approach using a pool of 20,000 bait probes designed to enrich regions with sequences similar to designed target gRNAs, with up to 5bp of mismatch to the 20bp spacer sequence. Using short-read sequencing on the probe-captured samples, we obtained high average sequencing depths of approximately 200 reads per probe site. A total of 64 *P. tremula x alba* and *P. tremula x tremuloides* trees containing 26 CRISPR/Cas9 insertion events, and 32 *E. grandis x urophylla* trees containing 11 CRISPR/Cas9 insertion events, were studied.

Using mutect2 somatic mutation analysis software and manual inspection, we found four *bona fide* non-target mutation sites. These occurred in nine *Populus* insertion events with constructs targeting the *AG1/AG2* gene and three events in *Eucalyptus* targeting the *LFY* gene. Surprisingly, these mutations were more distantly mismatched with the target gRNA sequence than expected, with mismatches ranging from 3-5bp from the target. No non-target mutations were found that closely matched non-target sites (1-2bp divergence), were in protein coding regions, nor were any suspected Cas9 based mutations observed in DNA unrelated to the target site. Mutation location relative to the PAM site in off-target mutations were distinct from on-target mutations, with off-target mutations occurring more distal to the PAM. Overall, rates of novel somatic variants within the surveyed sequences were similar to off-target mutations ($9x10^{-7}$ vs $7x10^{-7}$). Our results suggest that off-target mutations are a negligible source of risk.

We thank the USDA Biotechnology Risk Assessment Grant Program, grant # 2017-33522-27098, for financial support.

Key words: Mutation, forest, sterility, *AGAMOUS*, *LEAFY*, gene-editing