



Populus tremula x alba 717-1B4 leafy and agamous knockout trial (Year 8)



Populus tremula x tremuloides 353-53 leafy and agamous knockout trial (Year 8)

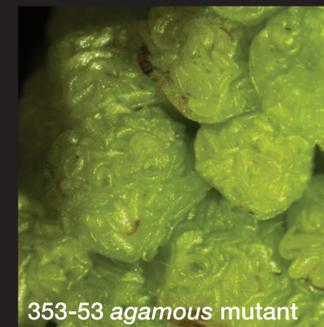
Observable but Highly Infrequent Off-Target Mutations In CRISPR/Cas9-Expressing Transgenic Trees Engineered for Containment

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353-53 (male) catkins



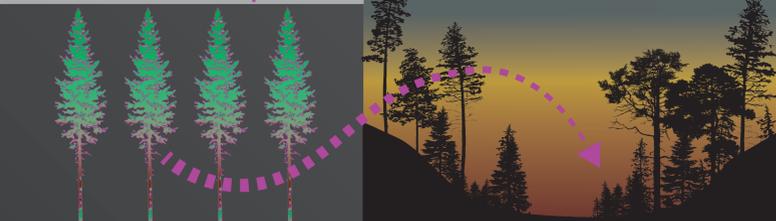
353-53 agamous mutant



P. alba 6k10 leafy mutant

Reproductive containment in forest biotechnology

Pollen or seed dispersal

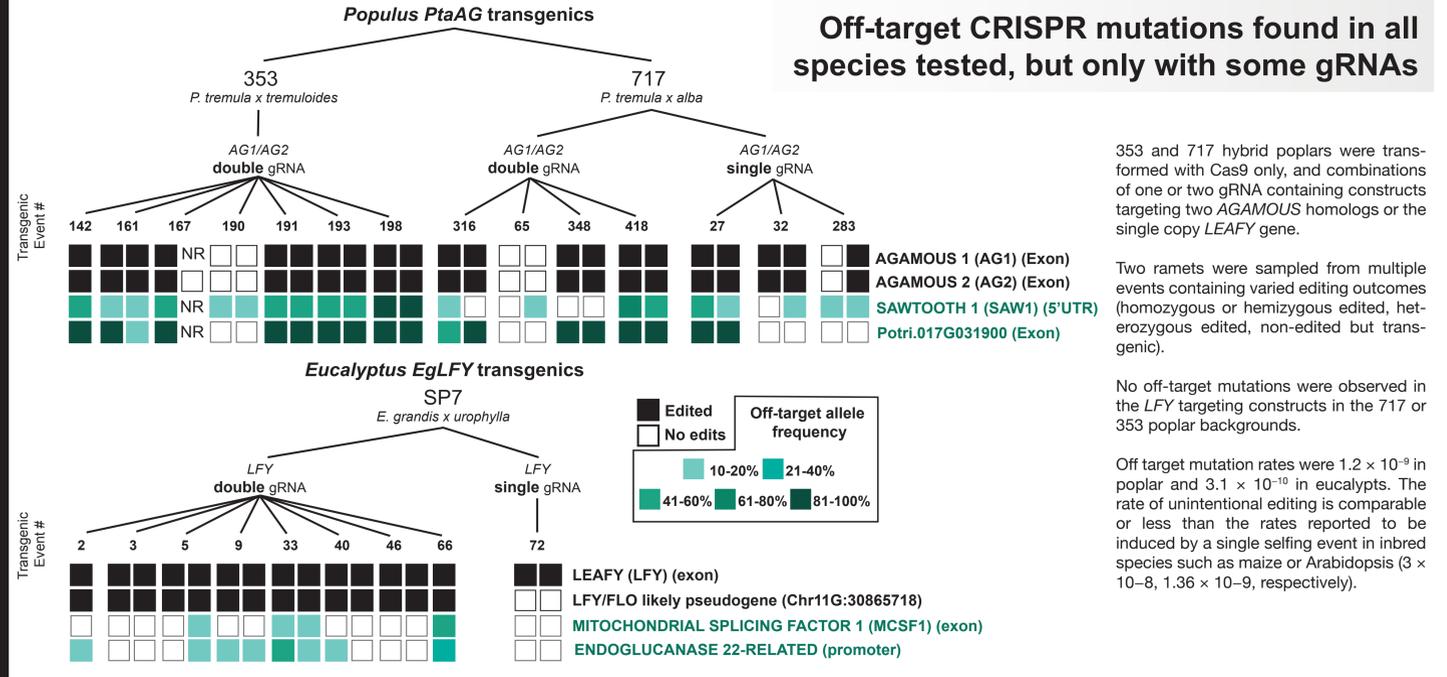
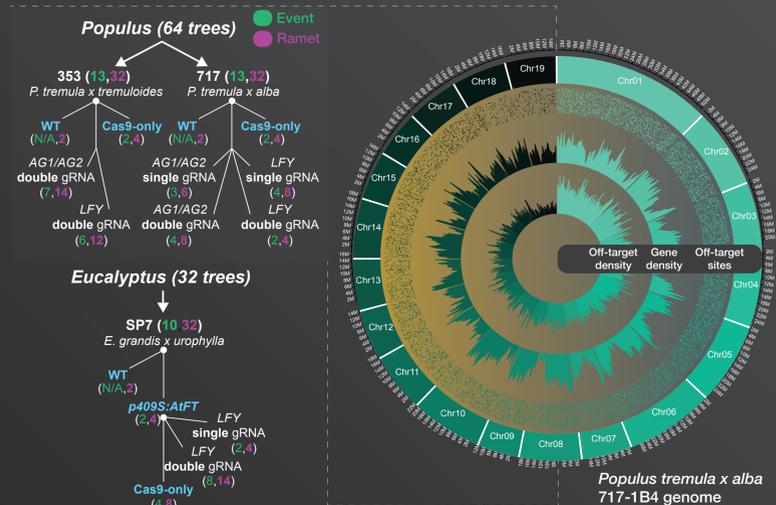


GE plantation

Native forest

GE trees with advantageous traits might share habitat with native trees with which they can interbreed, or encroach on native forests as exotics. Many trees are wind-pollinated or have wide seed dispersal, making containment more challenging than some other crops. We have used CRISPR/Cas9 to produce edited poplars and eucalypts with complete sterility, which was difficult to accomplish with previous transgenic approaches.

CRISPR/Cas9: a highly specific editor in plants... but what about when expressed for many years?



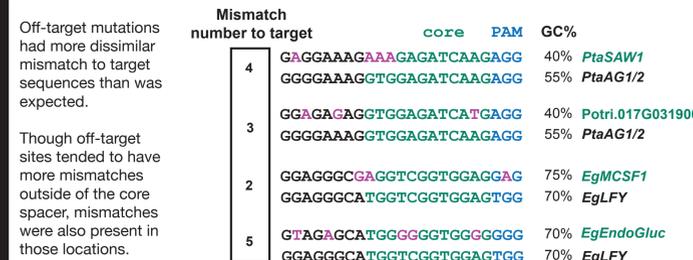
353 and 717 hybrid poplars were transformed with Cas9 only, and combinations of one or two gRNA containing constructs targeting two AGAMOUS homologs or the single copy LEAFY gene.

Two ramets were sampled from multiple events containing varied editing outcomes (homozygous or hemizygous edited, heterozygous edited, non-edited but transgenic).

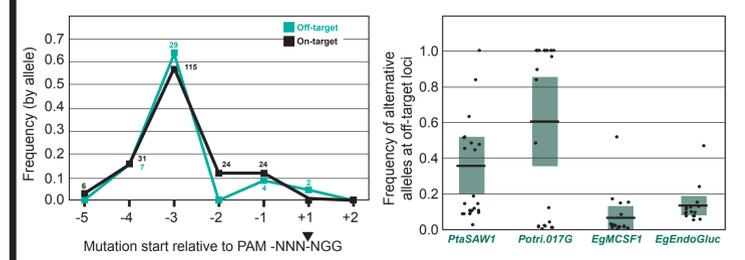
No off-target mutations were observed in the LFY targeting constructs in the 717 or 353 poplar backgrounds.

Off-target mutation rates were 1.2×10^{-9} in poplar and 3.1×10^{-10} in eucalypts. The rate of unintentional editing is comparable or less than the rates reported to be induced by a single selfing event in inbred species such as maize or Arabidopsis (3×10^{-8} , 1.36×10^{-9} , respectively).

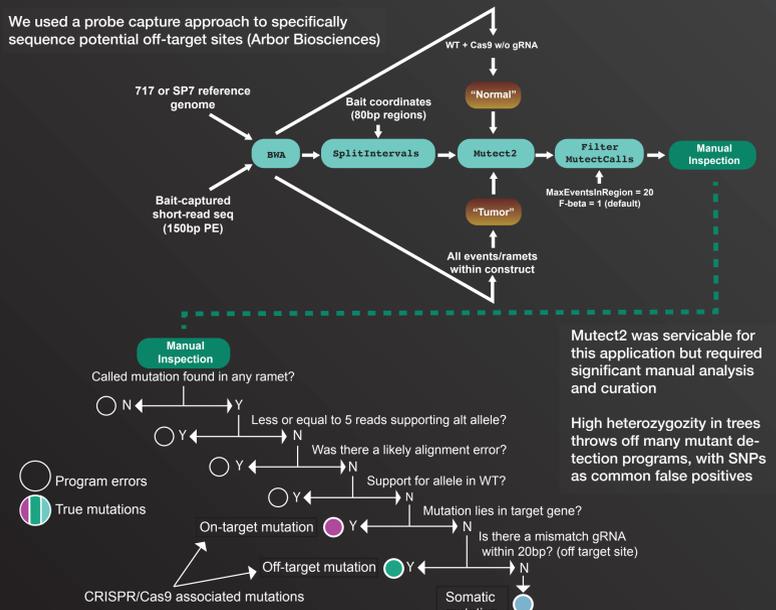
Off-target mutations had two to five bases of mismatch to target sequence



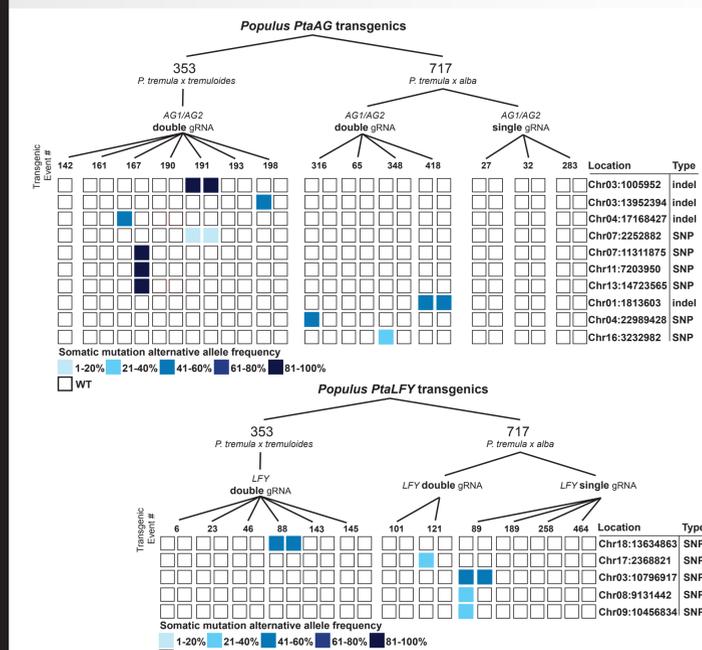
Mutations at off-target sites were PAM-proximal and varied in allele frequency



Targeted sequencing and detecting off-target edits



Somatic mutations were specific to single clones



Off-target mutants were confirmed using alternative sequencing approaches

Event	Ramet	Assigned score	Allele frequency	Nanopore coverage depth	Targeted sequencing coverage depth	
717 WT	11	WT	CACCAAGCCCCAAGAGAGAGGAAAGAAAGAGATCAAGAGGTCGCTATT	A1: 0.89	117	47
717 2AG #416	1	OT	CACCAAGCCCCAAGAGAGAGGAAAGAAAGAGATCAAGAGGTCGCTATT	A1: 0.84 A2: 0.06	398	45
353 2AG #196	3	OT	CACCAAGCCCCAAGAGAGAGGAAAGAAAGAGATCAAGAGGTCGCTATT	A1: 0.47 A2: 0.45	93	56

Off-target mutations were the same as found by targeted sequencing two years later

Conclusions and next steps

- Mutations due to CRISPR/Cas9 activity do occur and at sites for which *in silico* prediction algorithms place guides and off-target combinations at low priority.
- Shared off-target mutations between ramets suggests that off-target edits accumulate during transformation and tissue culture, not due to prolonged expression of Cas9. Future studies will untangle Cas9/gRNA expression from off-target editing.
- 717 and 353 edited poplars have been forming catkins and are in the final year of study (They will be culled next Fall, 2026). We hope to quantify the sterility phenotypes of knockouts for containment and any effects (or benefits) on vegetative growth.
- We thank the USDA Biotechnology Risk Assessment Grant Program, grant # 2017335227098 and 2023-33522-40414 for financial support. We also thank the Genetic Research on Engineering and Advanced Transformation of Trees (GREAT Trees) cooperative for their support.