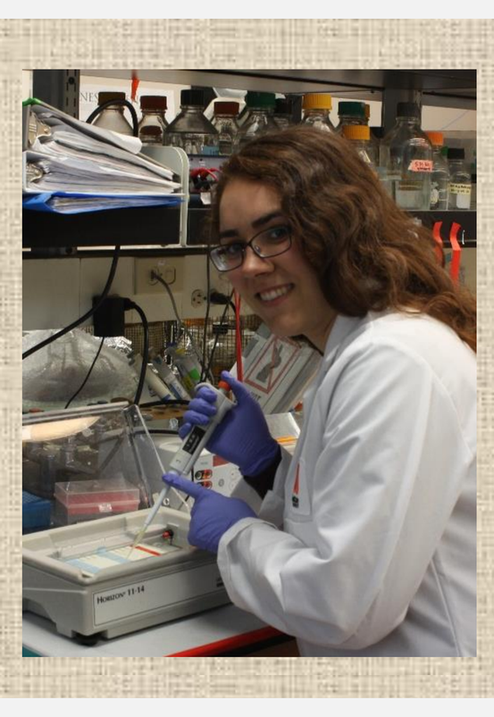

Knockout of Meiosis Gene in *Eucalyptus* using CRISPR/Cas9 for Genetic Containment

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Abstract

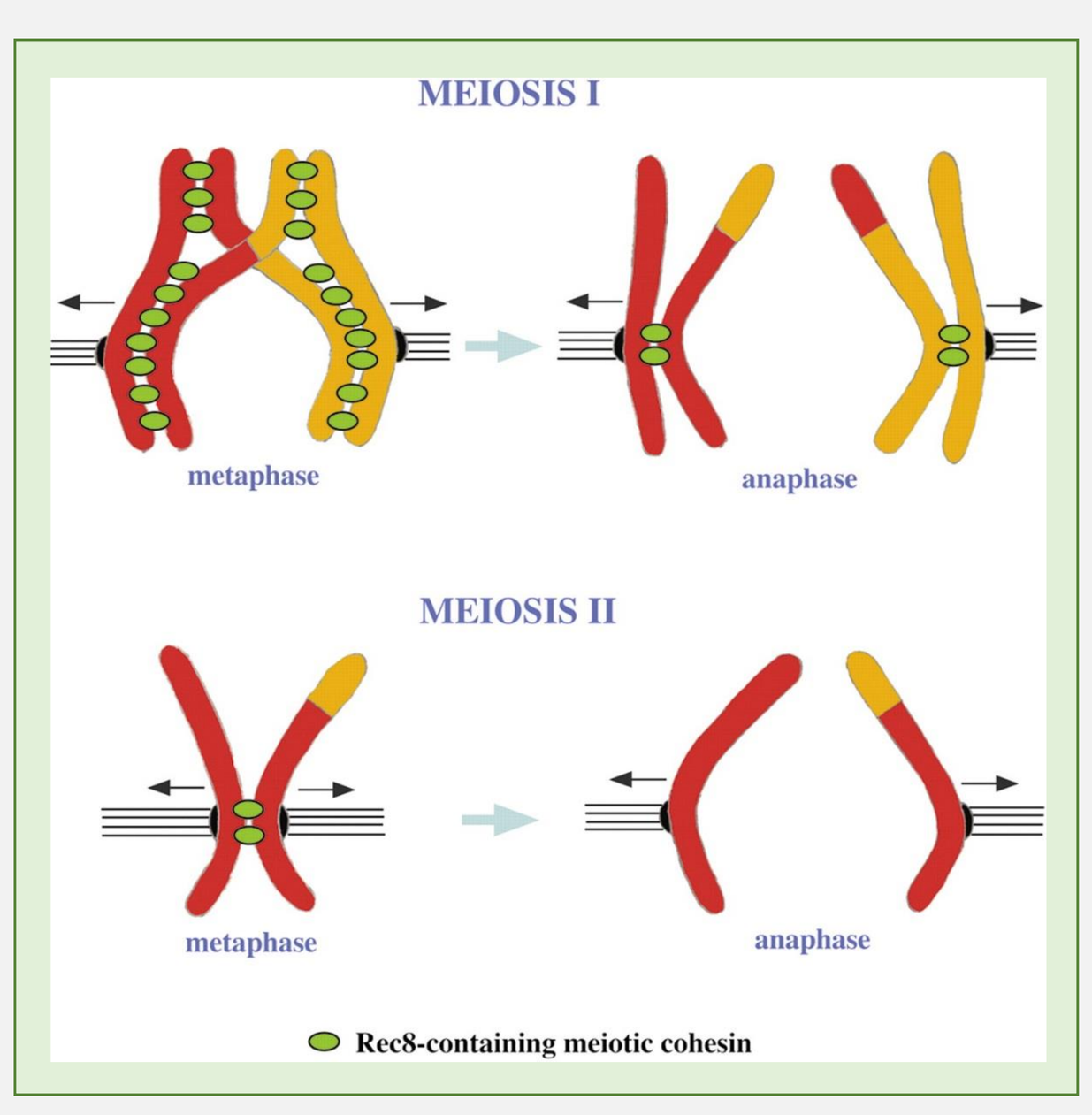
As a means for genetic containment we are using CRISPR/Cas nucleases to target the homolog of the meiosis gene *REC8* in eucalypt SP7 hybrid (*Eucalyptus grandis* × *E. urophylla*) to create bisexual sterile trees. Analysis of 12 independent transgenic events indicates high frequency of knockout (KO) mutations where ~67% were heterozygous biallelic KOs and 25% were monoallelic KOs. All of the plants with the active CRISPR locus had mutation in at least one allele. Seven events were selected for a greenhouse study to evaluate the effects on their growth and vegetative physiology. Further work will be done using an early-flowering *FT* overexpressed *Eucalyptus* genotype to analyze the floral phenotypes and fertility of *REC8* KOs.

Background

One of the Strauss Lab's main goals through the years has been to develop robust means of genetic containment of genetically engineered (GE) and exotic plantation trees through sexual sterility. Gene flow from GE trees to feral or wild populations is a concern for ecologists and regulatory bodies. Pollen has a wide geographic range it can travel over which makes gene migration especially concerning. This concern has been a barrier to wider use and acceptance of GE crops. Genetic containment that is efficient and reliable could overcome this barrier. Plantation trees like eucalypts are often vegetatively propagated so there is no commercial need for fertile plants and gene mutations that cause total sterility could provide effective genetic containment.

Gene Targeted: *SYNAPTIC 1/MEIOTIC RECOMBINATION PROTEIN8* (*REC8*)

- One of three novel eucalypt gene targets chosen after extensive bioinformatics and literature review in *Arabidopsis*, tobacco, and rice.
- Essential to meiosis (Cai et al. 2003, Zang et al. 2006)
  - Codes for a highly conserved across kingdoms basal cohesin protein in a cohesin complex that attaches sister chromatids
  - Cohesin complex must be cleaved for chromatid separation during the metaphase-to-anaphase transition (Kitajima et al. 2003)
- Chosen because there was only one copy of the gene in *Eucalyptus* and mutation of *REC8* orthologs in rice and *Arabadopsis* cause bisexual sterility and normal vegetative phenotypes (Bai et al. 1999, Shao et al. 2011)



Schematic of Rec8 in meiosis

Figure (Lee et al. 2003) shows role of the Rec8-containing meiotic cohesin (shown as green ovals) during chromatid separation in meiosis. We hypothesize that mutation to *REC8* will cause meiosis to not be completed.

CRISPR/Cas System used: Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR)/Cas

- Gene editing technology for site-directed modification
- Adapted from an anti-viral defense mechanism naturally occurring in bacteria and archaea
- Works by delivering guide RNA's complexed with the Cas9 enzyme into a cell where it cuts the DNA at the desired location

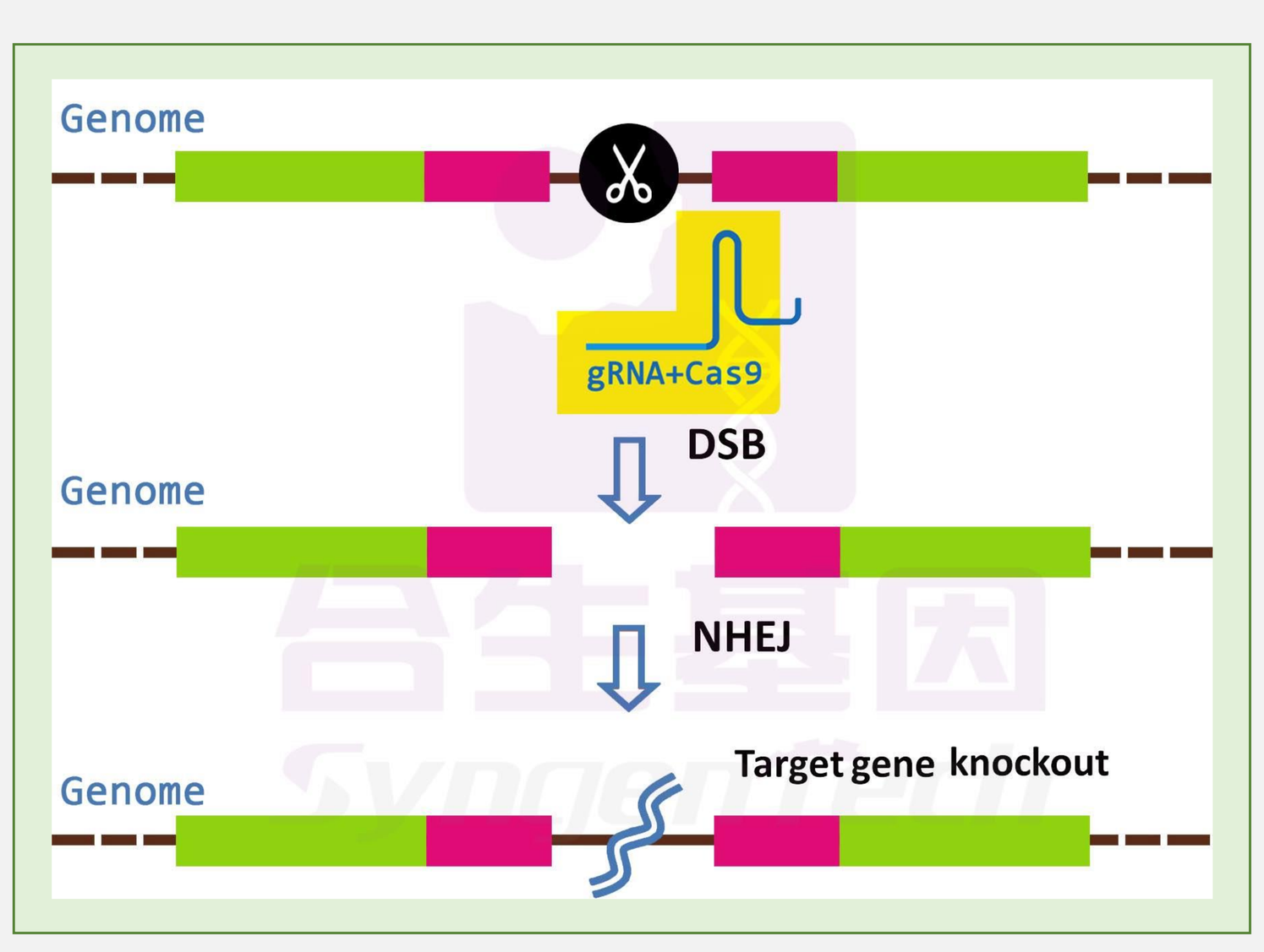
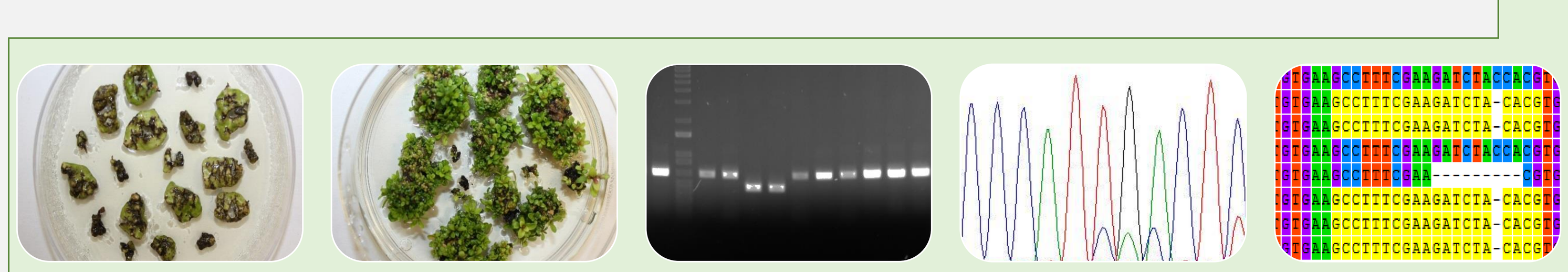


Illustration (SyngenTech) of how CRISPR can be used to knockout genes. Cas9 enzyme makes a double stranded break (DSB) at the target site complementing the guideRNA it is complexed with. Nonhomologous end joining (NHEJ) leads to indels when the break is repaired and can cause loss-of-function mutations in the target gene.

Methodology

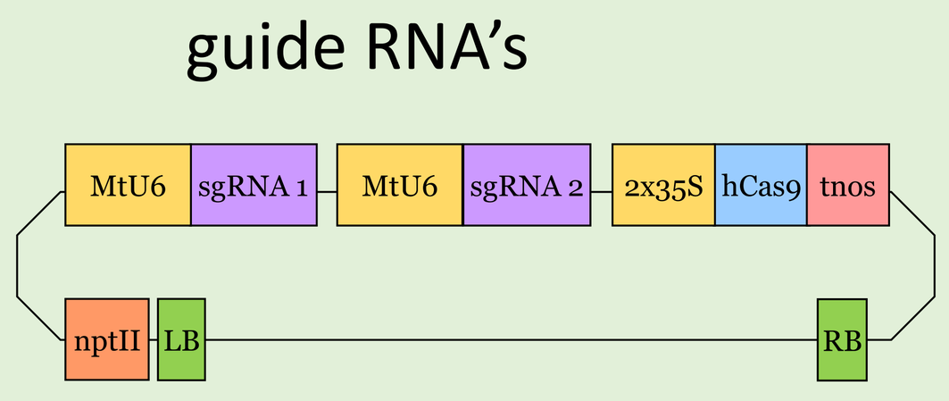
1. *Agrobacterium* mediated transformation using CRISPR constructs
2. Regenerate and grow plants
3. Extract DNA, confirm transgene using PCR and perform allele specific PCR for sequencing
4. Sanger sequence targeted gene sequence
5. Analyze alignments using MEGA6 to identify biallelic knockout mutants



Agrobacterium mediated transformation | Plant regeneration | DNA extraction for PCR analysis | Sanger sequencing | Mutation analysis

CRISPR/Cas9 Vectors

1) Construct with two guide RNA's



2) *CAS9* only control construct

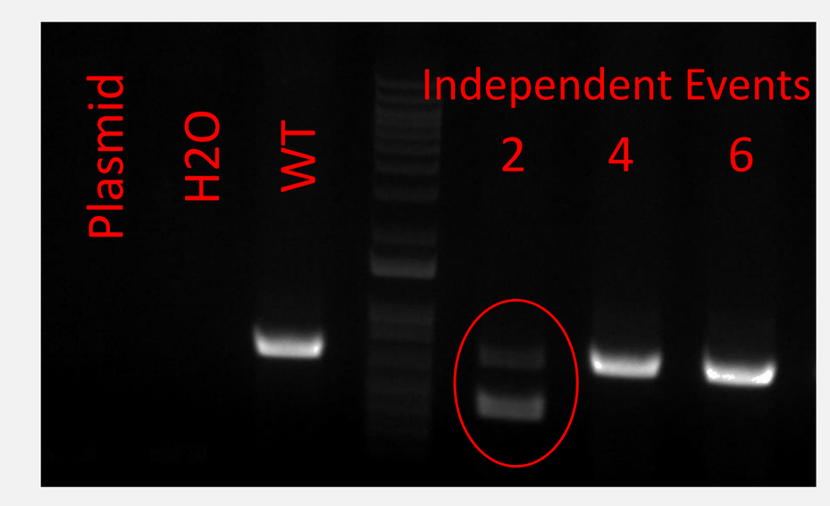
Alleles were identified using SNPs in the *Eucalyptus REC8* gene sequence

TGCTTGGAGGGTTGGATGAC TAAATTTGGAGGGACATCCATGTTAGGAGTTTCAGCTTTC
AGCCTTTTATGTTAAACAGTAACAATAAAAAAACTC(C/T)GATGAGACAATTCCGAGATGC
ATTTTCTTGCAGCGTACATGTGTGATGAACAGCATGAAGATCGCTCATCTCATGGAGCTAC
CACCAATAGTACTAGCATGTGGTTTACTGAATGAAAATAGAAGCATCTATTATCCTCCTCCTC
TCATGGAGCTGTGGACGAGAAGTATCCAGCCTCCCCATGACTCACCTTCTGGTCAGCCTTT
GGCAAGCCTCTTTTATAGCAATTTGTCGTGCTTGATAGAGTGCAACCCCAGTTCAAAATAA
TAACCGTTCTCCACAGGAAGAACATCCCCGCCCTTGCCACCAGAACCATCATCGTCATCCC
C(A/C) CCAGAGAGAGCATATTATCAAGATTTAGGGGCACGGGTATGCCTCTCATCTTTTTA
CTGGAGTACCTGATTGCAATGTGTGTGTCTGGACTCCGTCTGATGAA(G/A)TATTTGATTGT
GAAGCCTTTCGAAGATCTACACGTGGAGGTCAACCCACA(G/T)TCACAAGATGTCTCCATA
GAGAAGCTGCGGAATAATTTTGAAAATC(A/G)GGAAATGCCCCCGGAAATTTTCATGGAA
GGATTTAGAAACAATCTCATCCACAACAAGACAGCGGCAG

PCR Universal Primers
PCR Allele Specific Primers
Sequences matching sgRNAs
SNPS

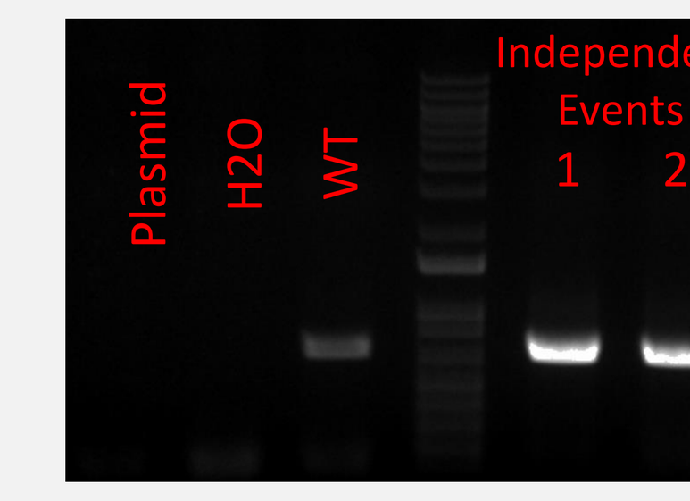
Results

REC8 heterozygote with large deletion



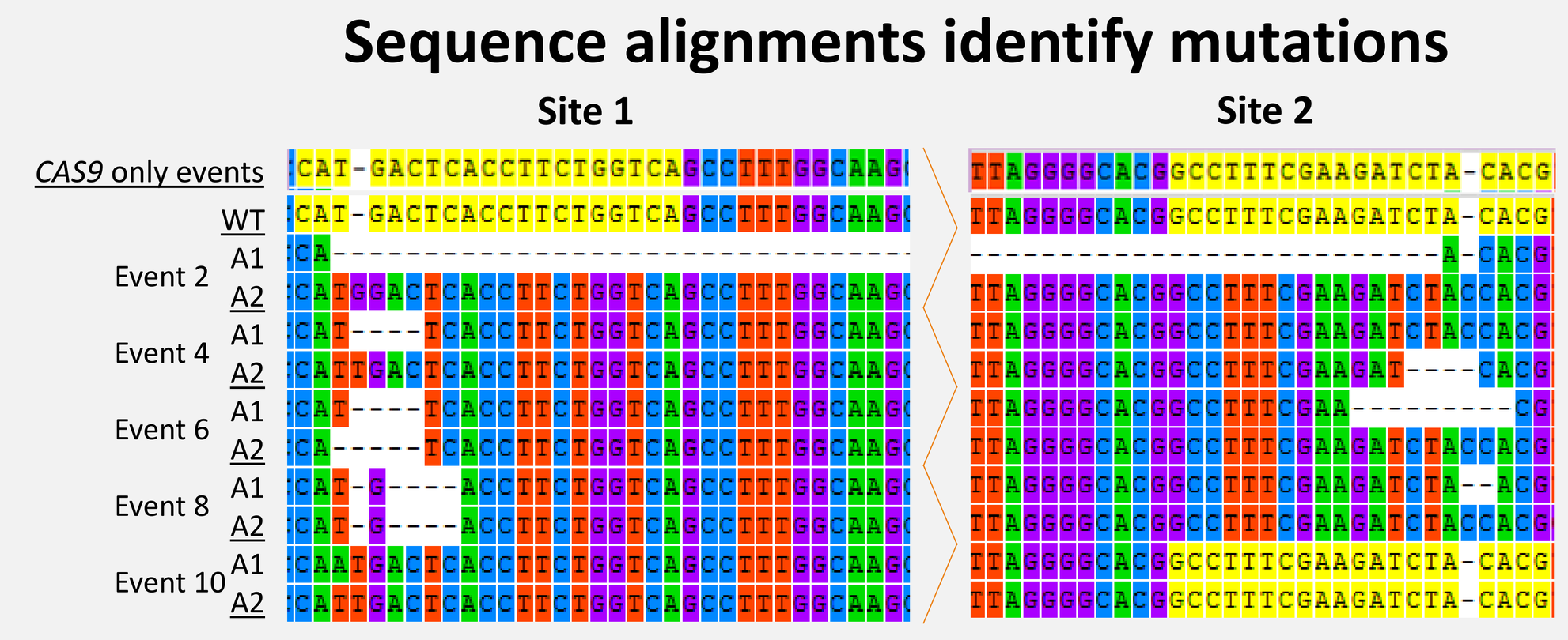
Gel image to the left shows three *REC8* independent events. Event 2 has two bands, one approx. same size as WT and one approx. 300 bp smaller, which indicate that it is a heterozygote with a large deletion.

Absence of mutations w/ *CAS9* only control events



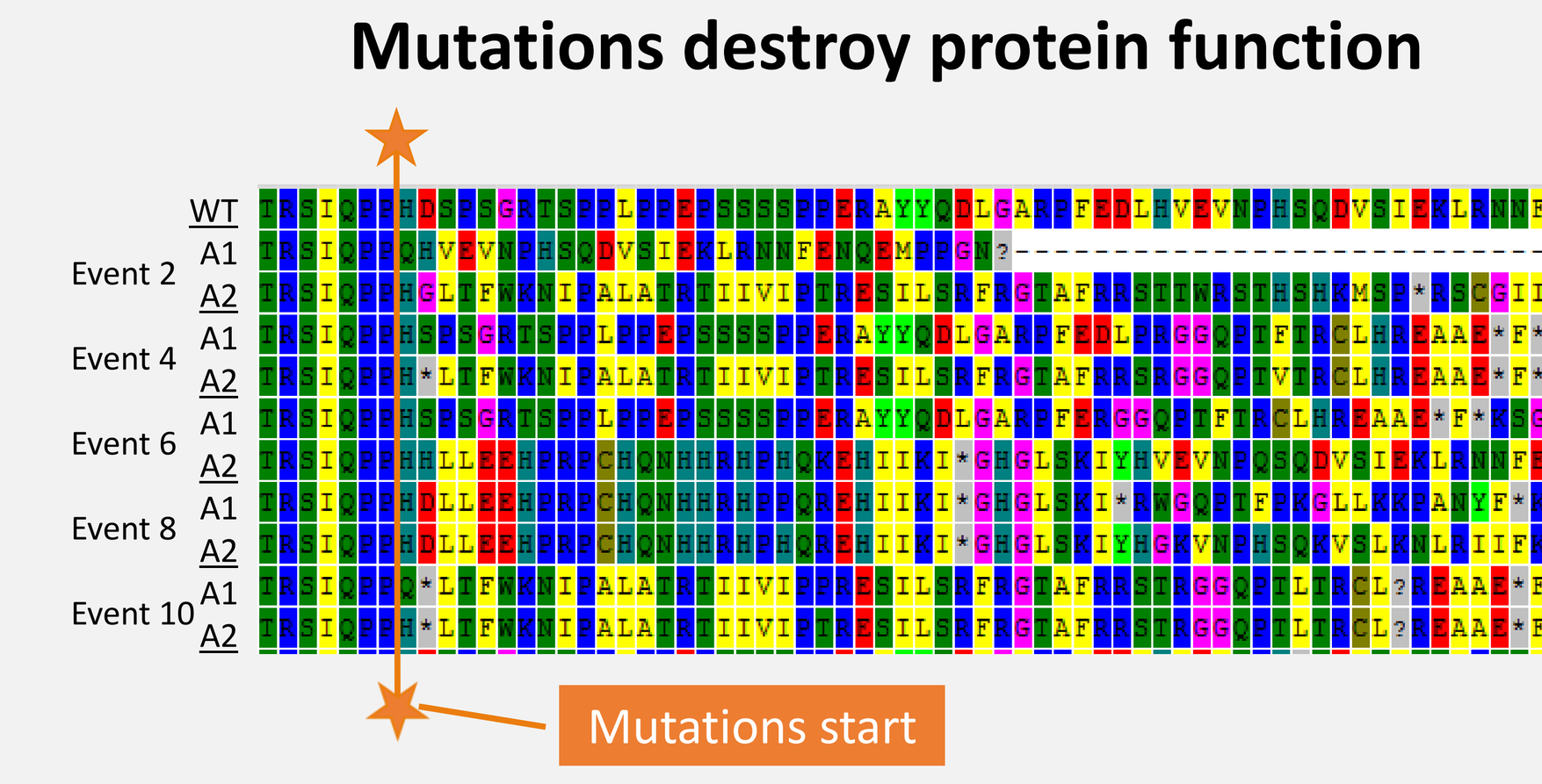
Gel image above shows two *CAS9* only vector control independent events. No obvious mutations observed based on band size.

Sequence alignments identify mutations



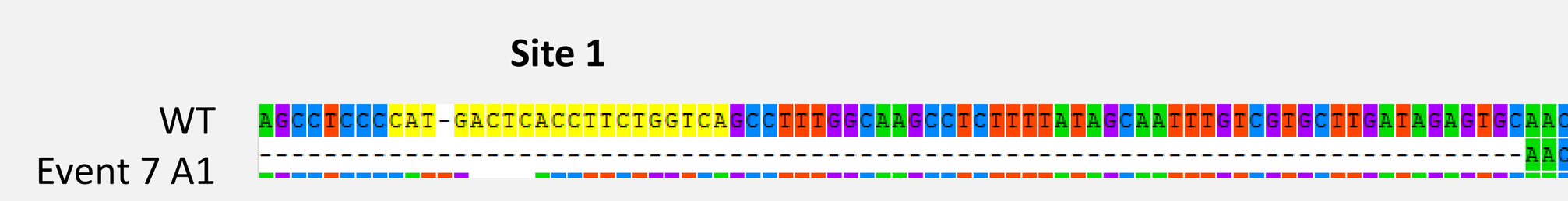
Sanger sequencing alignment of target sites 1 and 2 showing both alleles of independent events. WT target sequence is highlighted in yellow, transgenic events with yellow highlighted section had no mutation at that site. Event 2 allele 1 (A1) has a large deletion (-282 bp) while allele 2 (A2) and the other events shown have small insertions and deletions. *CAS9* independent events showed no mutations at either *REC8* target site

Mutations destroy protein function



Peptide sequences of the events are shown above. Mutations in the sequence can lead to frame shifts and early stop codons (denoted as *) that destroy protein function.

Undetermined allele in one event



One *REC8* event shown on the left had a large deletion (-171 bp) in allele 1 starting upstream of the 1st target site and no mutation at site 2. Allele 2 was undetermined; no amplicon was produced using the primers designed. Further study will be done to investigate why.

Most mutations lead to predicted loss-of-function KOs

An event was considered biallelic KO if neither allele had WT sequence and there was a large deletion or frameshift mutation. An event was considered monoallelic KO if one allele had WT sequence or a small non-frameshifting mutation.

		Sequence Modification		Peptide Modification	
Total Events	**Events w/both alleles defined**	**Heterozygous biallelic mutants**	**Monoallelic mutants**	**Biallelic KOs**	**Monoallelic KOs**
12	*11*	9 (75.0%)	2 (16.7%)	8 (66.7%)	3 (25.0%)

Small indels most common, Site 2 less active

Table shows mutation types at each site. Most prevalent type is highlighted in blue. Small refers to 15 bp or less.

		Mutation in each allele				
Total alleles	sgRNA	Small insertion	Small deletion	Large deletion	No mutation	Undefined
24	**Site 1**	9 (37.5%)	9 (37.5%)	3 (12.5%)	2 (8.3%)	1 (4.2%)
	Site 2	5 (20.8%)	5 (20.8%)	2 (8.3%)	11 (45.8%)	1 (4.2%)

Further Work

- Conduct randomized greenhouse study using 7 selected biallelic *REC8* knockout events, 10 *CAS9* only events, and WT
- Study effects on floral phenotypes using early-flowering *FT* overexpressed *Eucalyptus*
- Investigate putative large deletion in undetermined allele

Micropropagated plants in tissue culture.

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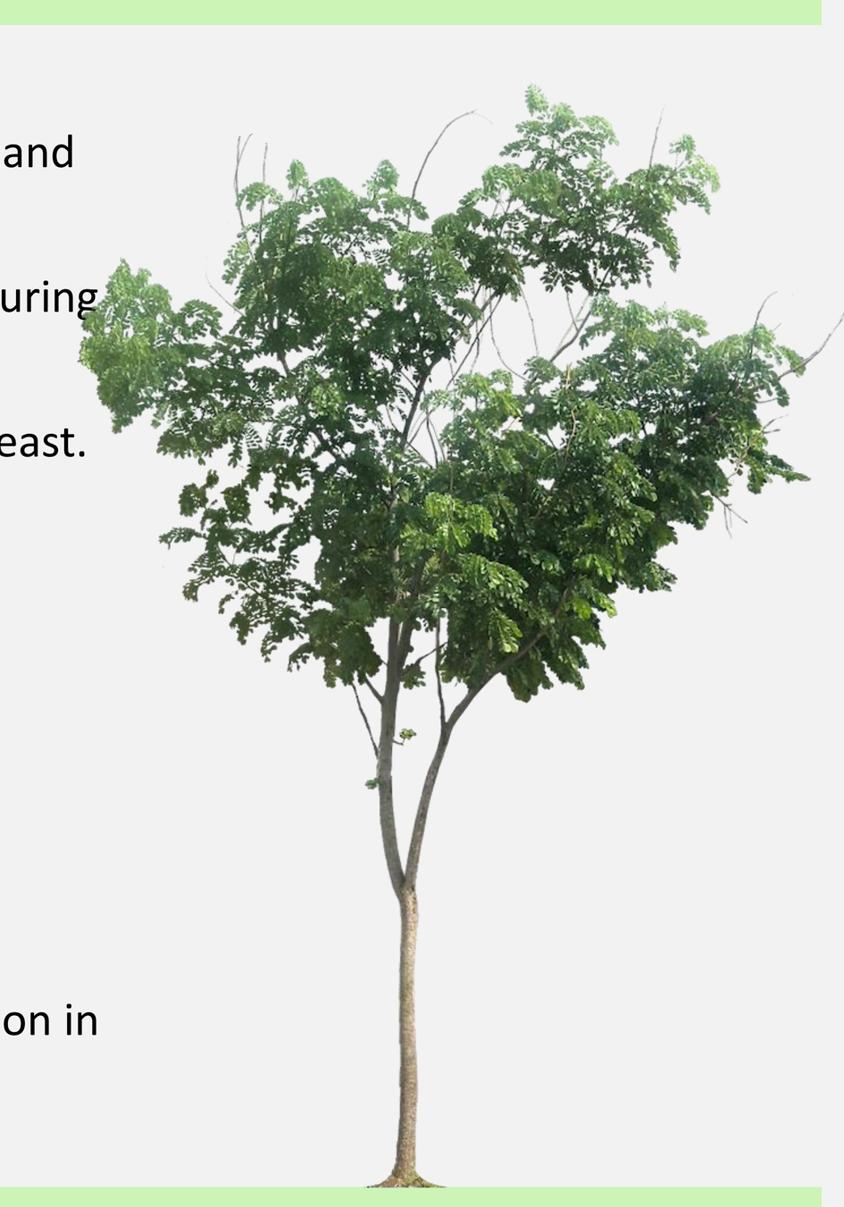

Acknowledgements

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