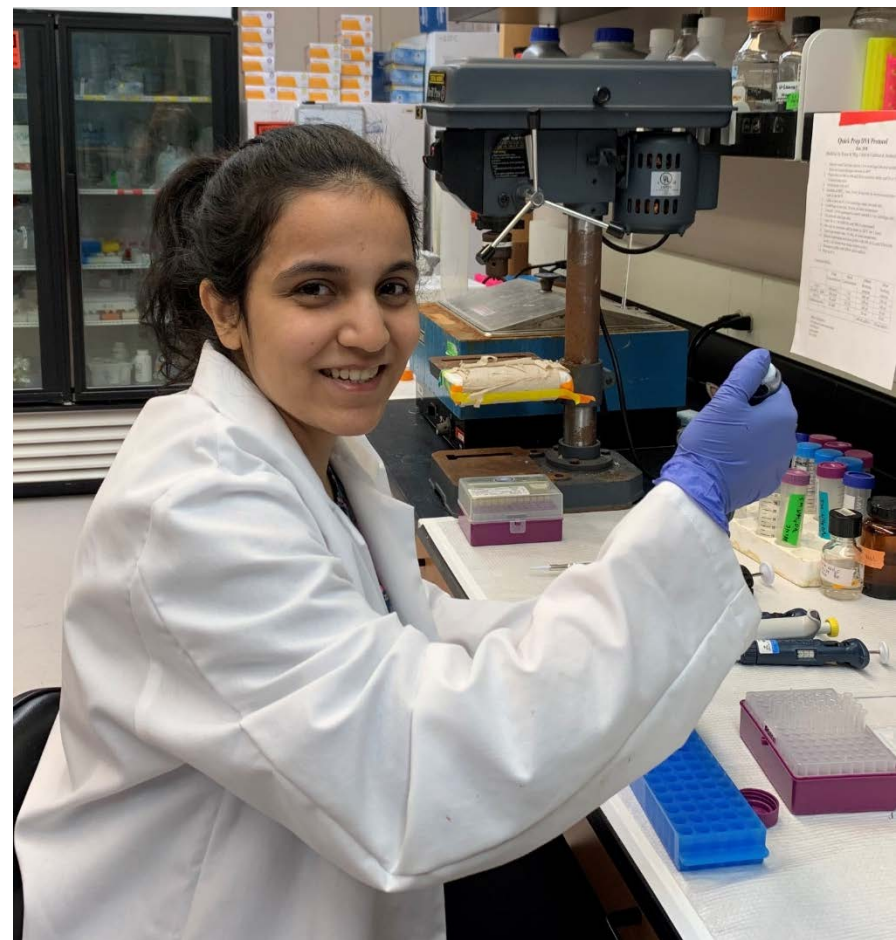# CRISPR and better trees: Gene editing to promote containment of exotic and genetically engineered Eucalyptus

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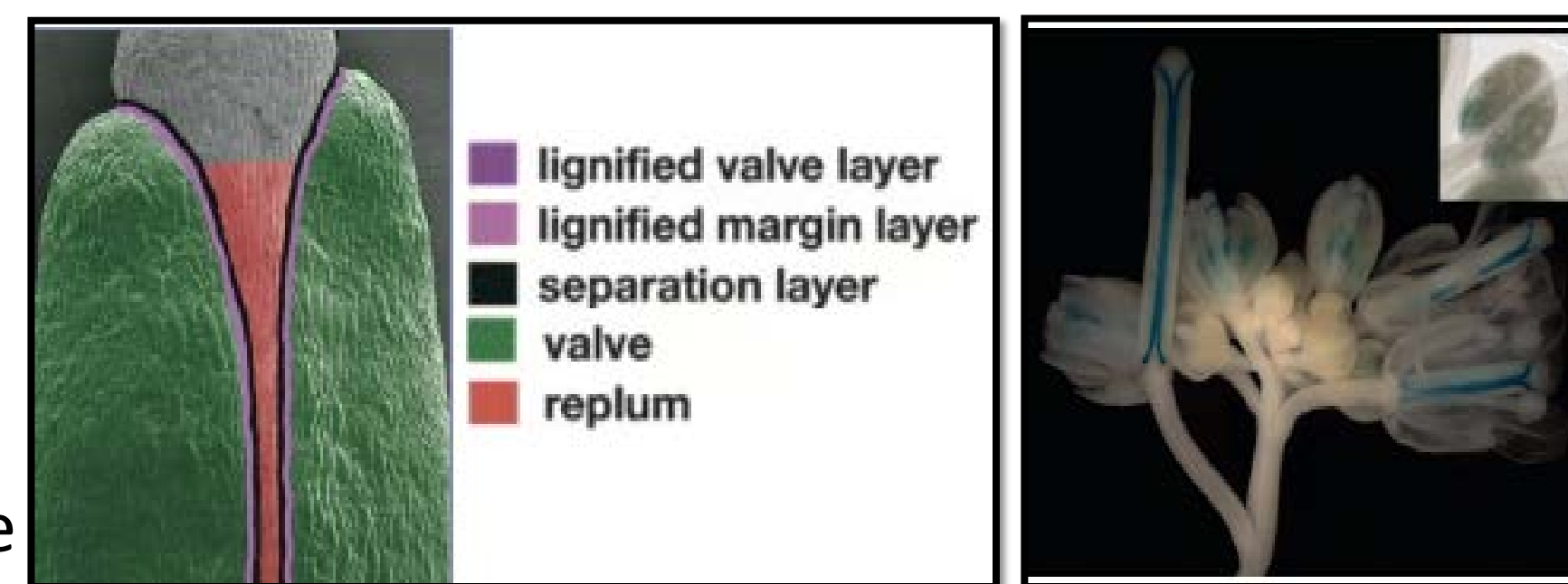
Oregon State University

Abstract

A major impediment to use of exotic and bioengineered trees is their propensity for spread by pollen and/or seeds. We have been using CRISPR-Cas9 gene editing as means to impart stable genetic containment when this is desirable from social (markets, regulation, public opinion) or ecological perspectives. Our studies focus on induction of loss-of-function mutations in two genes expected to be essential for male or female sterility. For these studies we transformed Eucalyptus, one of the most widely planted, productive, and sometimes invasive forest tree species in the world. We report a high rate of biallelic mutation—including the occurrence of frequent large deletions among guide RNA targets—for each of two reproductive genes, and an absence of visible vegetative morphological defects. Randomized studies of vegetative and reproductive phenotypes are underway.

Genes Targeted For Sterility

The Eucalyptus homologs of *Arabidopsis* genes were identified by sequence alignment and comparison of tissue specific-expression patterns. We studied:

1. **EDA 33: EMBRYO SAC DEVELOPMENT ARREST 33**
   - Helps in differentiation of three layers of the seedpod required for fruit dehiscence.
   - A non-functional *EDA 33* gene may induce female sterility.

- lignified valve layer
- lignified margin layer
- separation layer
- valve
- replum

https://doi.org/10.1016/S0092-8674(04)00217-X

A schematic of seed pod structure, and GUS staining to demonstrate the localization of *EDA 33* gene expression to valve margins in Arabidopsis (Liljegren 2004)

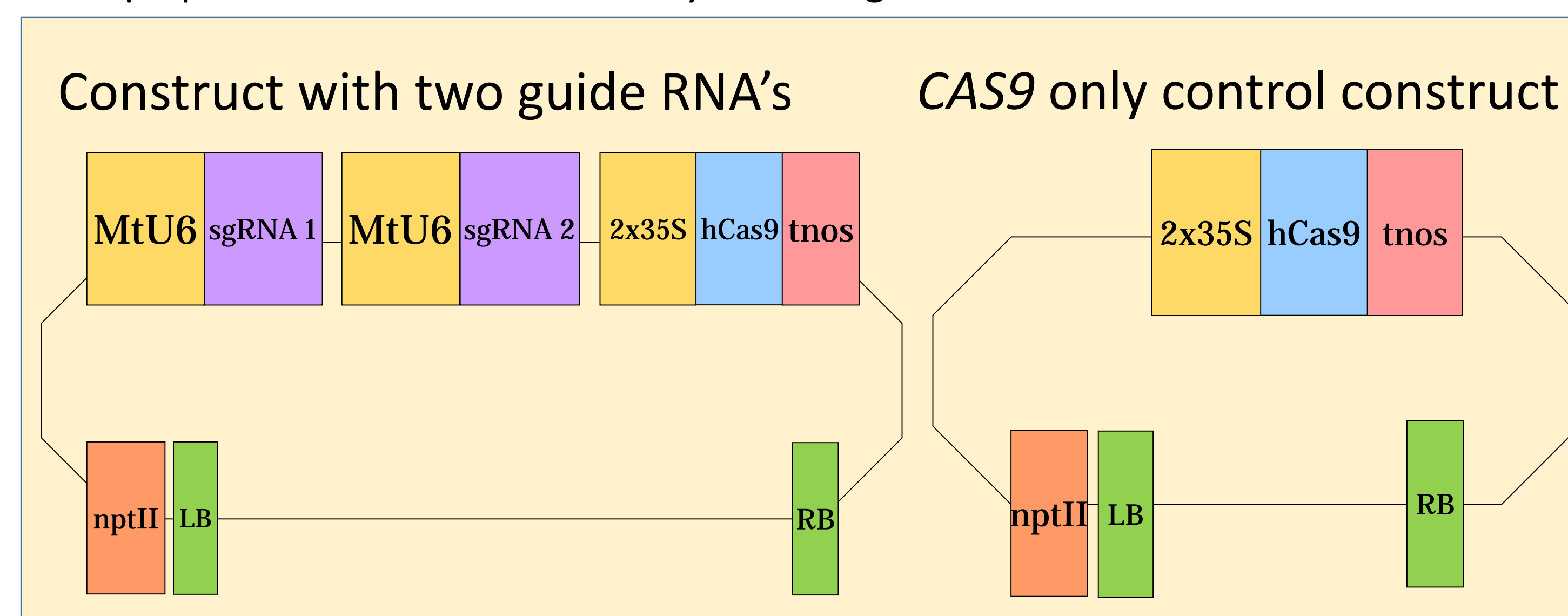2. **TDF1: TAPETAL DEVELOPMENT AND FUNCTION 1**
   - Plays a crucial role in anther development by providing enzymes for callose dissolution, materials for pollen wall formation, and essential nutrients.
   - A defect in *TDF1* may provide male sterility.

https://doi.org/10.1111/j.1365-313X.2008.03500.x
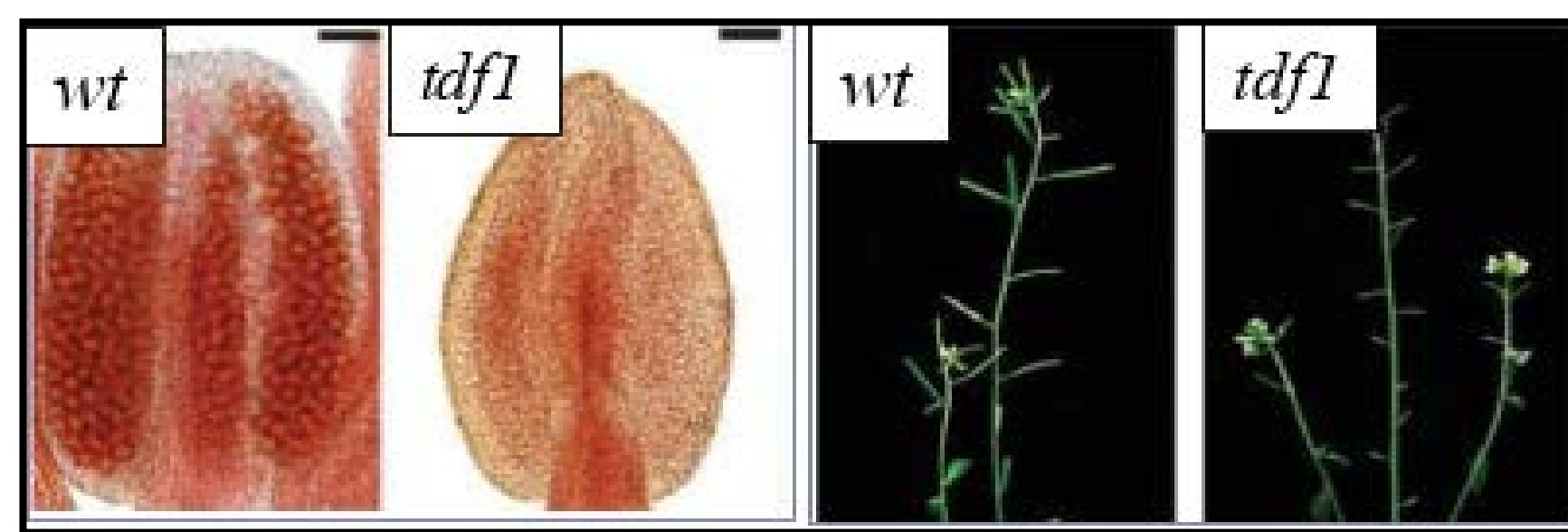
Arabidopsis anthers are shown. No viable pollen (dyed red) was produced in the *tdf1* mutant.

Siliques in the *tdf1* mutant were small and contained no seed.

Experimental Vectors

CRISPR-Cas9 (Clustered regularly interspaced short palindromic repeats) gene editing technology was used for creating frameshift or deletion mutations target gene DNA site). Control vectors had only Cas9 and no guide RNAs, and mutation vectors had two guide RNAs directed to sites 5' in the gene and approximately 50-100 bp apart. Both were inserted by stable Agrobacterium transformation.

Construct with two guide RNA's

| MtU6 | sgRNA 1 | MtU6 | sgRNA 2 | 2x35S | hCas9 | tnos |

| nptII | LB | | RB |

CAS9 only control construct

| 2x35S | hCas9 | tnos |

| nptII | LB | | RB |

Experimental Overview

Step 1 -Target gene selection and genomic analysis

Step 2 – Design of CRISPR-Cas 9 construct

pGCK-2010-Fwd (4002 bp)

Step 3 – Agrobacterium transformation

Step 4 –Plant DNA extraction and allele-specific PCR amplification

Step 5 – Sanger sequencing to obtain nucleotide sequence

Step 6 – Greenhouse analysis of growth and flowering

Guide RNAs and Allele-Specific PCR Primers

EDA33 nucleotide sequence

ATGGACTTCAACCAAAGCAAGTTCTCGAACAACTTTTGGGATCTTGGCTTGG
GCATGGAAGATCAAACCCTCCATTCCAATGACCAACATCATCATCAGCCGCCTT
TCACTTCCCTTTGGCCTAGCATCCATCTCCCATTAATGCACCAAACAACTCCAA
CTACTTCCCAGATTCCATCTTCCCACTTTGTGAATGATAGTAGCATTGGGGTTG
TGGCAAATCA**AATCGAAGACAAGGATGAAGA**(G/A)CCTGAAGAGGAGCTTG
GAGCCATGA**GGGAGATGATGTACAAGATTGCGG**(T/C)GATGCAACC(G/A)GT
(T/C)GACGT(T/C)GACCCGACGACGATTCGGAAGCC(A/G)AAGAGGCGGAA
TGTCCGGATCAGTGATGATCCTCAAAGTGTAGCGGC(G/A)CGCCT(T/C)CGGC
GGGAAAGGATAAGCGAGAAGATCAGGA**TCCTCCGAGGCTCGTCCCCGGGG**
GGAC(A/G)AAGATGGACACGGCTTCGATG**T**TGGACGAGGCTATTCGCTATGT
CAAGTTCTTGAAGCGACAAATCCGTTTGCTTCAACAGCCAAATAACCAAAACC
CTACACCAGATCCGGGCGCAGTCACGGGGAGCTTGGTGGGAGGGGATTGGC
AAGGCGTGATTGCACCGAGCAAACCACCCTCCACTACCTCGTCATCGTCCTTC
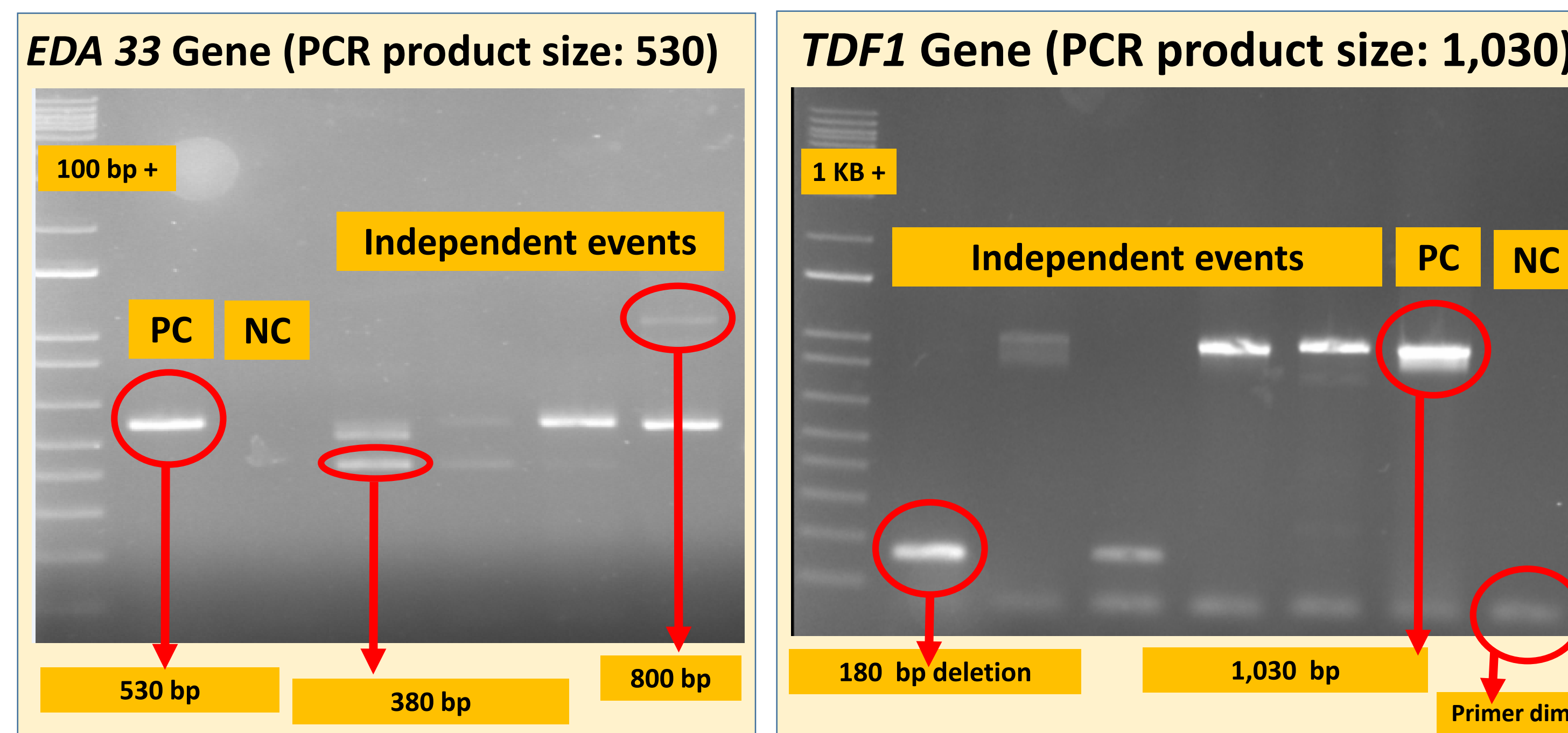GAAGCACAGGTCGGATCAAGGTTTGGGTTCTGGTCCAATGGTGGTGGTGGT

- Yellow: Allele specific Primer location – Forward and reverse
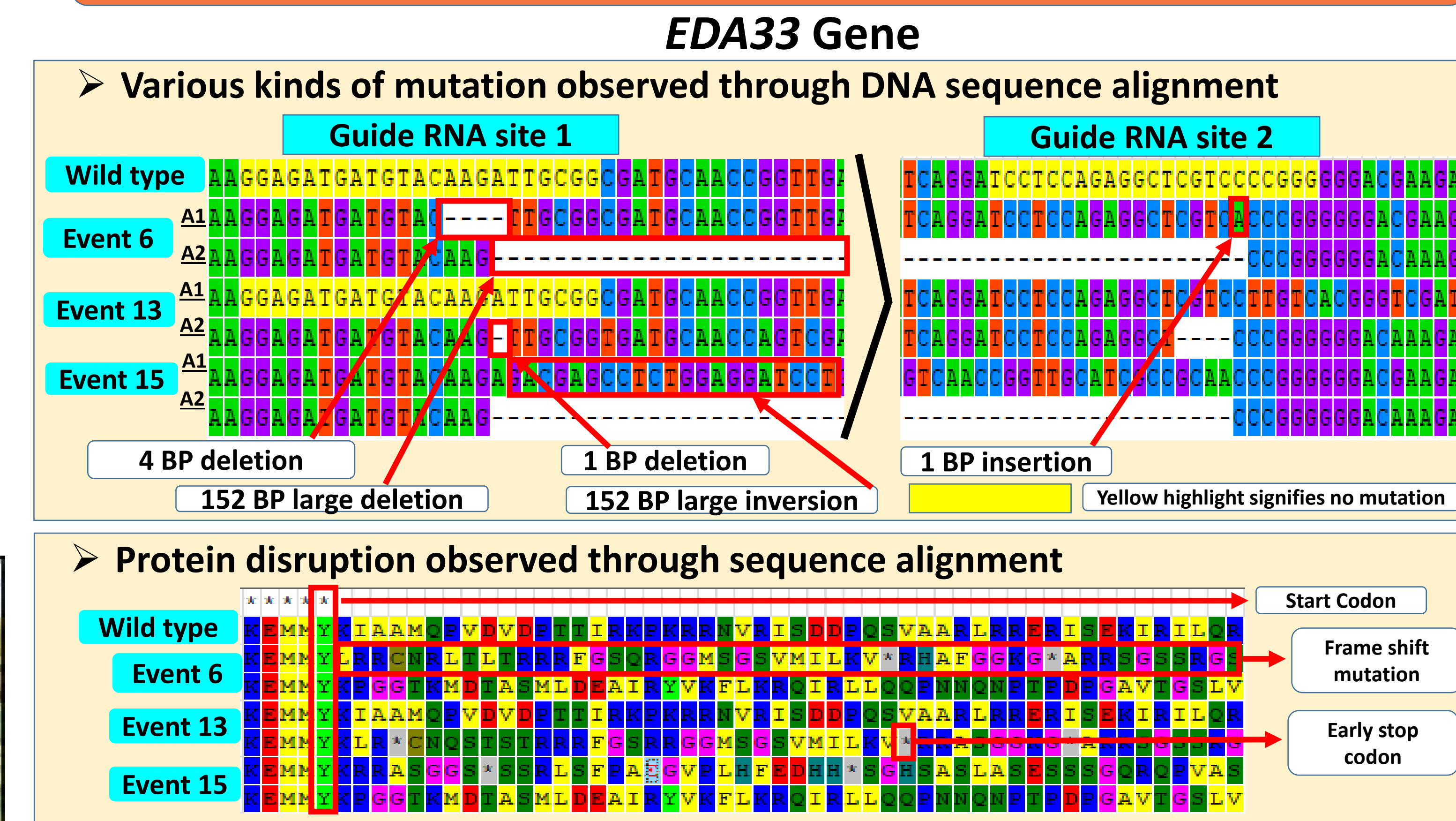- Cyan: Two of the guide RNA sequence
- **(T/C)** Red highlighted letters signifies SNP presence to aid in allele-specific-PCR

The same procedure for allele specific primer design or SNP identification was used for *TDF1*, (not shown)

Large CRISPR-Induced Deletions

EDA 33 Gene (PCR product size: 530)

100 bp +

Independent events

PC | NC

530 bp | 380 bp | 800 bp

TDF1 Gene (PCR product size: 1,030)

1 KB +

Independent events | PC | NC

180 bp deletion | 1,030 bp | Primer dimer

Examples of Mutations Observed

EDA33 Gene

- Various kinds of mutation observed through DNA sequence alignment

Guide RNA site 1 | Guide RNA site 2

Wild type
Event 6 — A1, A2
Event 13 — A1, A2
Event 15 — A1, A2

- 4 BP deletion
- 152 BP large deletion
- 1 BP deletion
- 152 BP large inversion
- 1 BP insertion
- Yellow highlight signifies no mutation

- Protein disruption observed through sequence alignment

Wild type
Event 6
Event 13
Event 15

- Start Codon
- Frame shift mutation
- Early stop codon

DNA sequence and protein sequence alignment for *TDF1* gave similar results (not shown).

High Mutation Rates

EDA33

Total events studied	Heterozygous biallelic knock-out rate	Homozygous biallelic knock-out rate	Monoallelic mutant rate
13	10 (76%)	2(20%)	1(7%)

CRISPR-cas9 has been highly successful in creating INDEL mutations. Mutation ranged from large deletions (-152 bp) to large insertions (+530 bp). Two events also had an inversion of 152 bp.
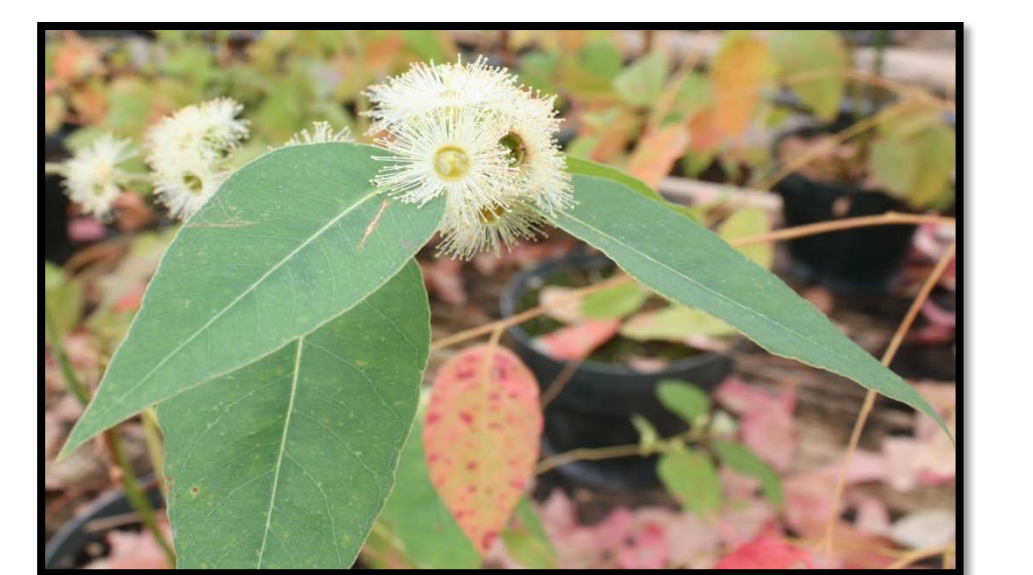
EDA33 mutant
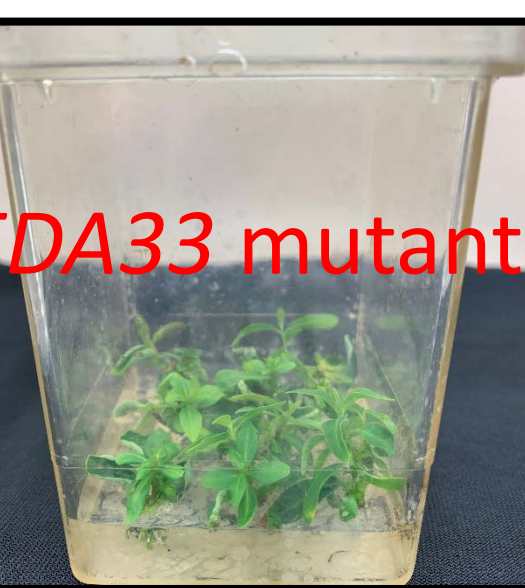
TDF1

Total events studied	Heterozygous biallelic knock-out rate	Monoallelic mutant rate	No mutations observed
14	6(43%)	8(57%)	0

CRISPR-cas9 has been moderately successful in creating INDEL mutations. The knockouts mainly had a large deletion (-900 bp) in one allele whereas small deletions of 4-5 bp in the other allele.

TDF1 mutant

Future work

- Five knock-out events, a similar number of Cas9-only controls, and wild type trees are being propagated for a randomized greenhouse study
- A similar number of early flowering Eucalyptus knock-out events are being propagated for a randomized greenhouse study

Acknowledgements