LEAFY knockouts in *Eucalyptus* have normal vegetative growth and lack stamens and carpels

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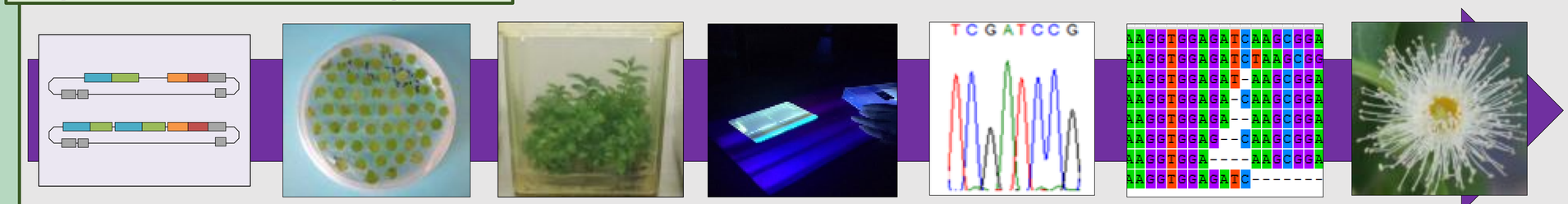
Abstract

To promote public, market, or regulatory acceptance of exotic or genetically-modified eucalypts, we have been developing options for genetic containment. Clustered Regularly Interspace Short Palindromic Repeats (CRISPR) nucleases are highly efficient at inducing knockout mutations in target genes. Thus, mutating key floral development genes may be effective at creating permanently sterile trees unable to spread via pollen and/or seed. We found very high mutation efficiency (~97% of transgenic plants produced were biallelic knockouts) using three CRISPR-Cas9 constructs that targeted one or two loci within the Eucalyptus ortholog of *LEAFY* (*EgLFY*). Two transgenic populations were generated; one using a normal flowering *Eucalyptus grandis x urophylla* hybrid (WT SP7), and another using two early-flowering (*AtFT* overexpression) SP7 genotypes previously transformed with *AtFT*. All normal-flowering SP7 CRISPR plants showed normal vegetative development in the greenhouse, including those biallelic *EgLFY* knockouts. The early-flowering knockouts produced indeterminate and sterile floral shoots without stamens or ovules, whereas transgenic plants without *EgLFY* knockouts had phenotypically normal flowers and floral organs. No mutations were detected in 12 transgenic controls that contained Cas9 but no sgRNAs. CRISPR-Cas9 directed against the eucalypt *LFY* gene appears to be a highly efficient means for generating sexually contained eucalypts.

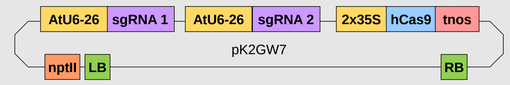
Steps for CRISPR-Cas mutagenesis



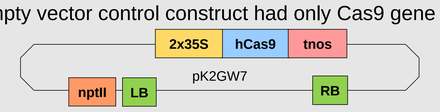
Construct design and cloning | *Agrobacterium*-mediated transformation | Event selection and regeneration | Target site amplification and gel extraction | Sanger sequencing | Event sequence analysis | Growth and fertility study in the greenhouse

CRISPR Cas9 constructs

Experimental constructs had one or two guide RNAs (pictured)



Empty vector control construct had only Cas9 gene

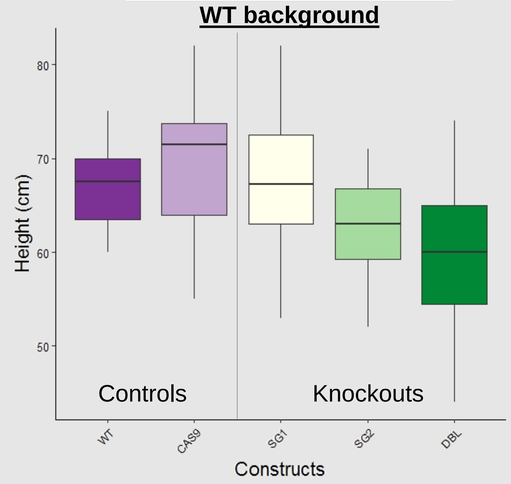


CRISPR Cas9 construct had no detectable effects on growth or morphology

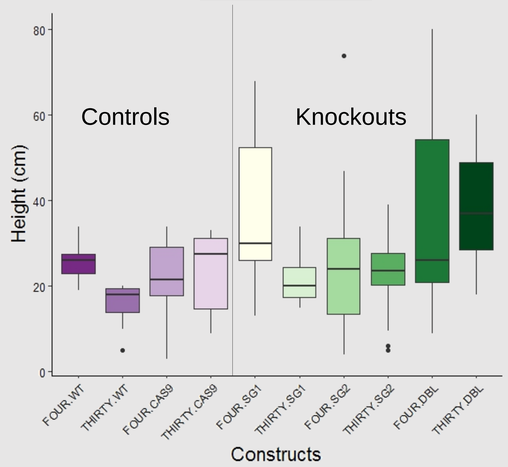
All ramets from one KO event with WT background

All ramets from one KO event with *FT* background

Height versus construct with WT background



Height versus construct with *FT* background



Apart from the marked differences among the *FT*-early flowering and non-*FT* populations (top vs. bottom images, excepting non-*FT* WT in lower image), there was no evidence (P>0.05) that transformation, CRISPR construct, or target knockout had an effect on height or mean diameter (not shown) within populations.

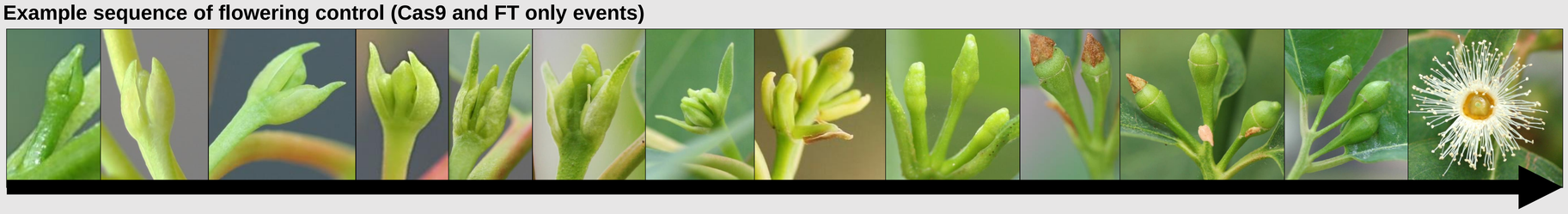
High mutation rate

Population	Total events	Mutation type	# events	Mutation freq.
FT LFY-CRISPR	60	Biallelic KO	58	97%
		WT	2	3%
WT LFY-CRISPR	10	Biallelic KO	10	100%
		WT	0	0%
All CRISPR	**70**	**Biallelic KO**	**68**	**97%**
		WT	**2**	**3%**
FT Cas9 control	10	Biallelic KO	0	0%
		WT	10	100%
WT Cas9 control	2	Biallelic KO	0	0%
		WT	2	100%

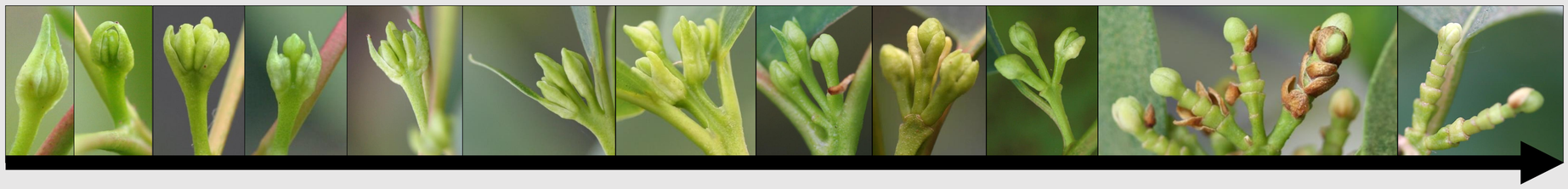
Mutation rates were calculated after sequencing both alleles for *EgLFY* separately using allele-specific primers.

Developmental sequence from bud formation to flower

Example sequence of flowering control (Cas9 and FT only events)

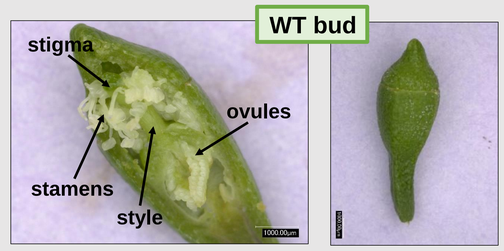


Example sequence of *EgLFY* knockouts

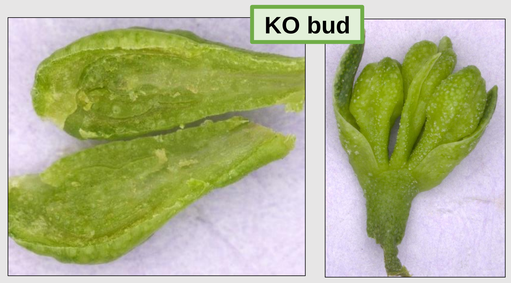


No ovules/stamens seen in KOs

WT bud



KO bud



Summary

- CRISPR Cas9 nucleases are highly efficient at inducing mutations in endogenous genes of eucalypts
- The reproductive whorls of loss-of-function knockouts of *EgLFY* remain vegetative
- Growth was not affected by the presence of CRISPR Cas machinery
- No mutations were seen in 12 Cas9 control events

Acknowledgements

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