CRISPR-Cas9 mutagenesis for genetic containment of forest trees

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BACKGROUND

- Field research and commercial use of transgenic plants are severely limited by regulations, and associated ecological and legal risks, for which transgene dispersal are major elements
- These concerns warrant an efficient, reliable, and bisexually-effective method for genetic containment of vegetatively propagated transgenic trees and other perennial crops
- The **C**lustered **R**egularly **I**nterspaced **S**hort **P**alindromic **R**epeats (CRISPR)/Cas9 gene editing system is revolutionizing reverse genetics studies in all systems including trees
- CRISPR/Cas9 will allow directed mutation of genes essential for sexual fertility, potentially enabling the production of predictably and reliably sterile trees

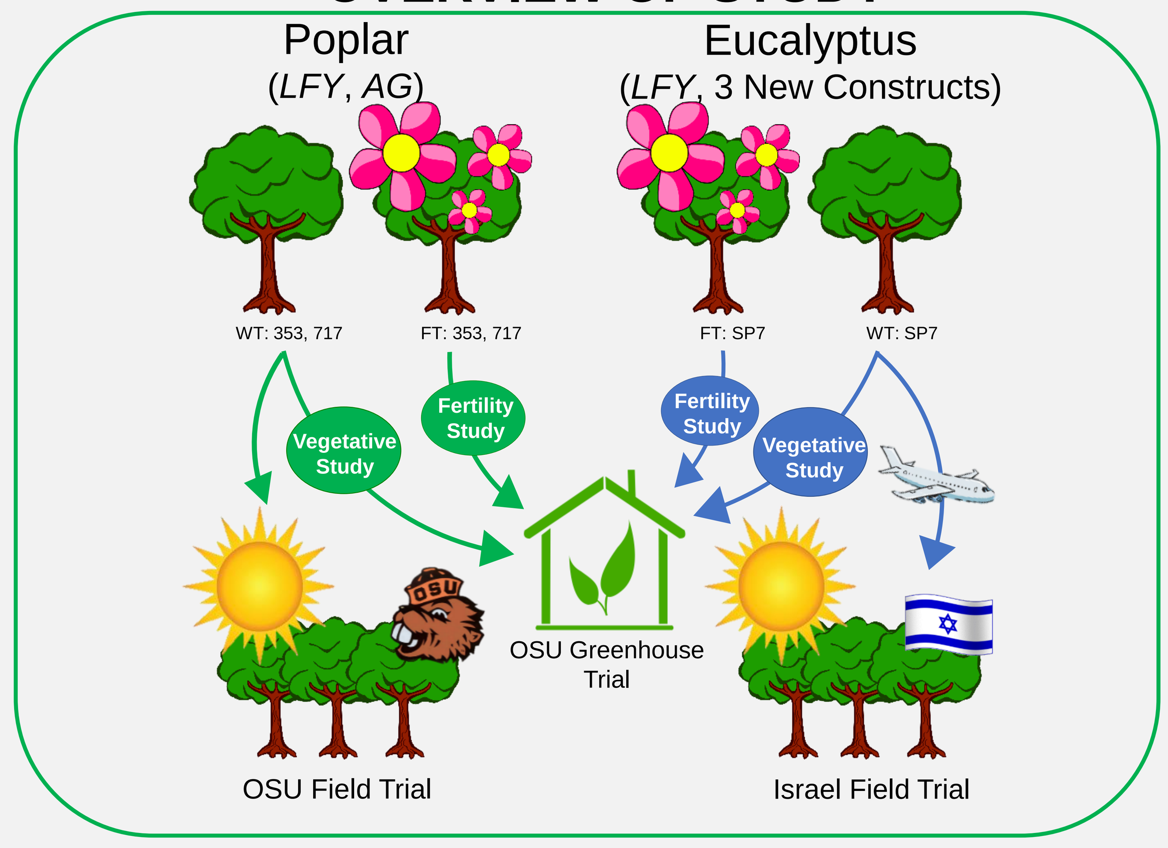
AIMS

- Investigate efficacy & stability of modified floral developmental genes as tools for mitigating or preventing transgene spread using CRISPR/Cas9
- Study the frequency of off-target mutagenesis in CRISPR transgenics
- Study methods of site-specific excision system of CRISPR/Cas9

APPROACH TAKEN

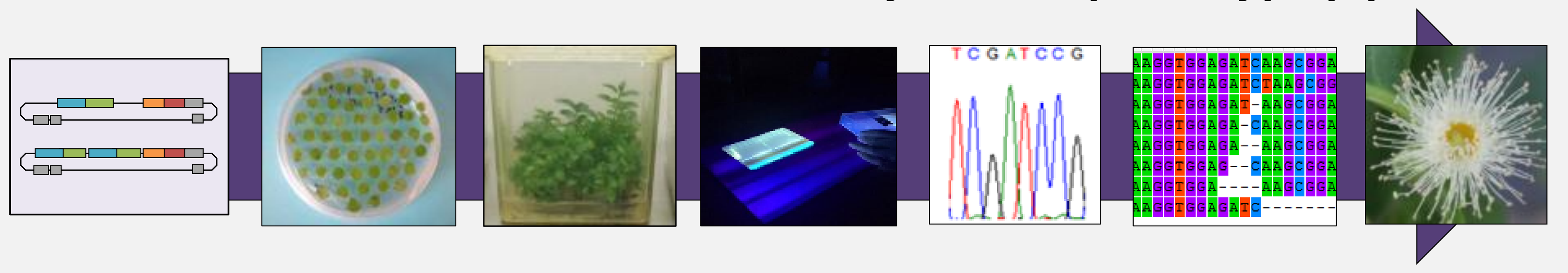
- Selected a total of five genes that are essential to normal flowering (*AG, LFY, EDA33, REC8,* and *TDF1*)
- Re-transformed *FT*-early flowering eucalypts with CRISPR to speed analysis of fertility
- Re-transformed *LFY* and *AG* knockout with *FT* gene to speed fertility analysis
- Performed greenhouse studies to assess fertility (*FT* events) and growth/morphology
- Established poplar field trial in USA and plan to establish eucalypt field trial in Israel
- Will study off-target mutagenesis in CRISPR transgenics using targeted bait-capture methods
- Will develop excision systems for removal CRISPR locus using meristem-specific promoter

OVERVIEW OF STUDY



Wild-type and early flowering *Eucalyptus* and *Populus* used in this study: 717, *Populus tremula* x *P. alba*; 353, *P. tremula* x *P. tremuloides* & SP7, *Eucalyptus grandis* x *E. urophylla*

CRISPR transformation, mutation analysis, and phenotype pipeline



Vector construction — Cocultivation and transformation — Plant regeneration — DNA extraction and PCR amplification — Amplicon sequencing — Sequence analysis — Growth and flowering assessment

CRISPR causes a very high rate of bi-allelic knockouts in eucalypt and poplar

Eucalypts	Total events	Mutation	# events	Frequency
FT LFY-CRISPR	60	Biallelic KO	58	97%
		WT	2	3%
FT Cas9 control	10	Biallelic KO	0	0%
		WT	10	100%
SP7 LFY-CRISPR	10	Biallelic KO	10	100%
		WT	0	0%
SP7 Cas9 control	2	Biallelic KO	0	0%
		WT	2	100%
All eucalypt	70	Biallelic KO	68	97%
		WT	2	3%

Poplar	Total events	Mutation	# events	Frequency
LFY-CRISPR	294	Biallelic KO	195	66%
		WT	99	34%
AG-CRISPR	194	Biallelic KO	162	84%
		WT	32	16%
Cas9 control	50	Biallelic KO	0	0%
		WT	50	100%
All poplar	488	Biallelic KO	357	73%
		WT	131	27%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ORIGINAL RESEARCH
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Variation in Mutation Spectra Among CRISPR/Cas9 Mutagenized Poplars

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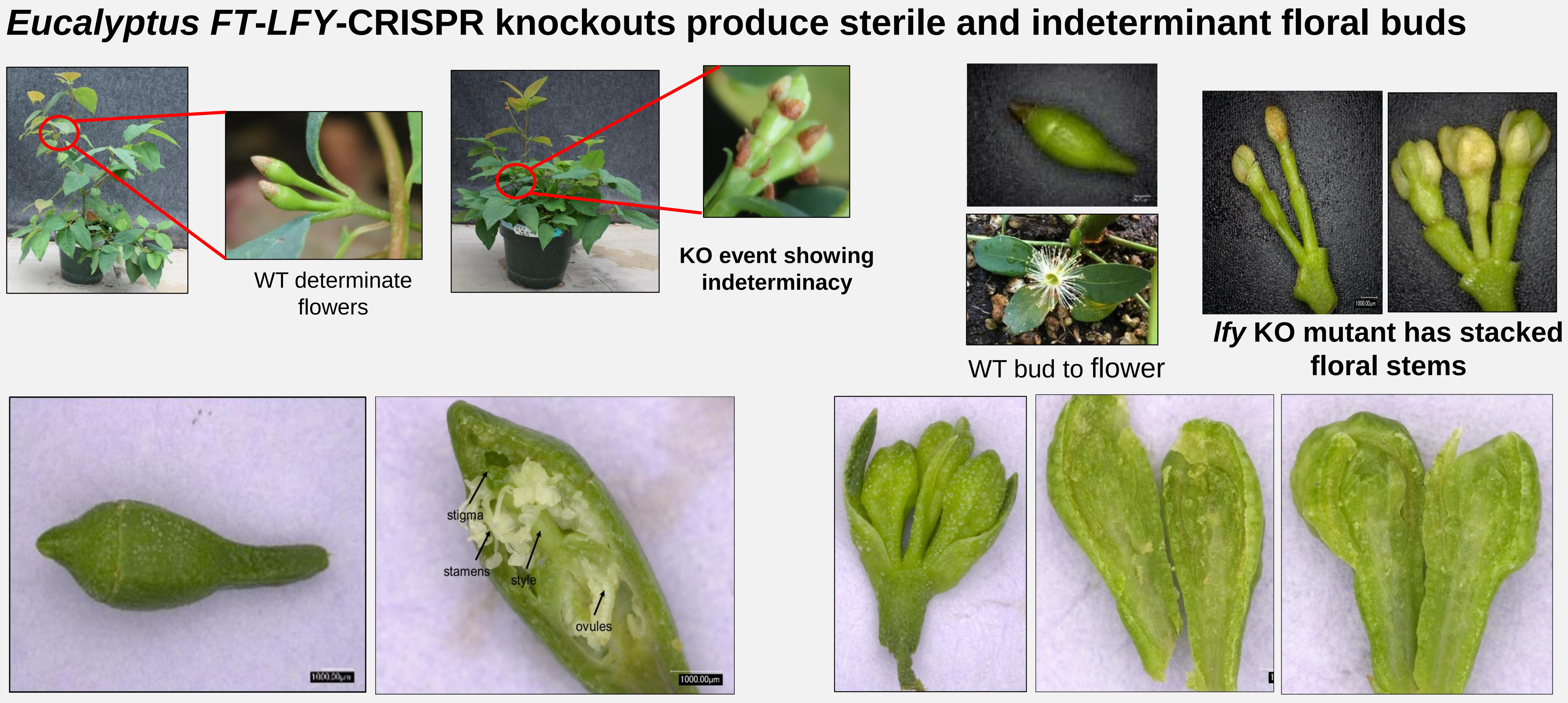
CRISPR eucalypts & poplars grow well in greenhouse experiments

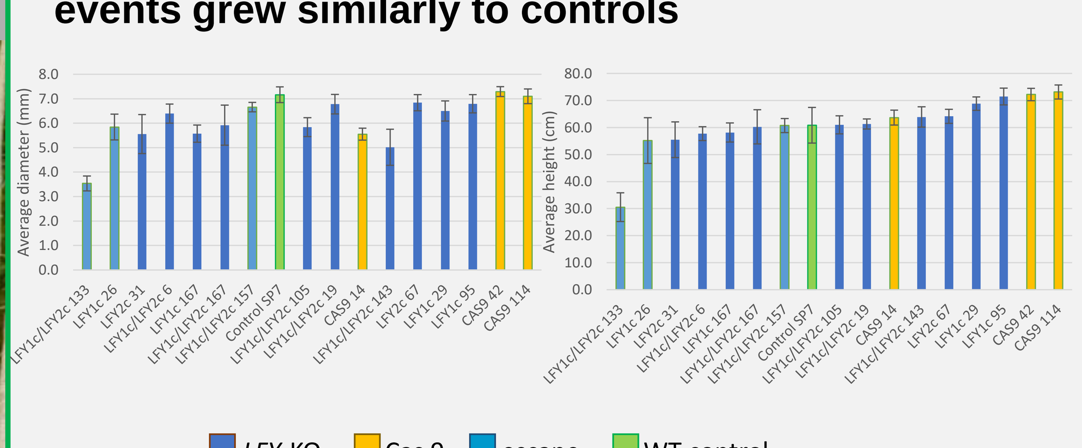
Eucalyptus FT LFY-CRISPR — *Eucalyptus SP7 LFY*-CRISPR — *Populus* CRISPR

Eucalyptus FT-LFY-CRISPR knockouts produce sterile and indeterminant floral buds

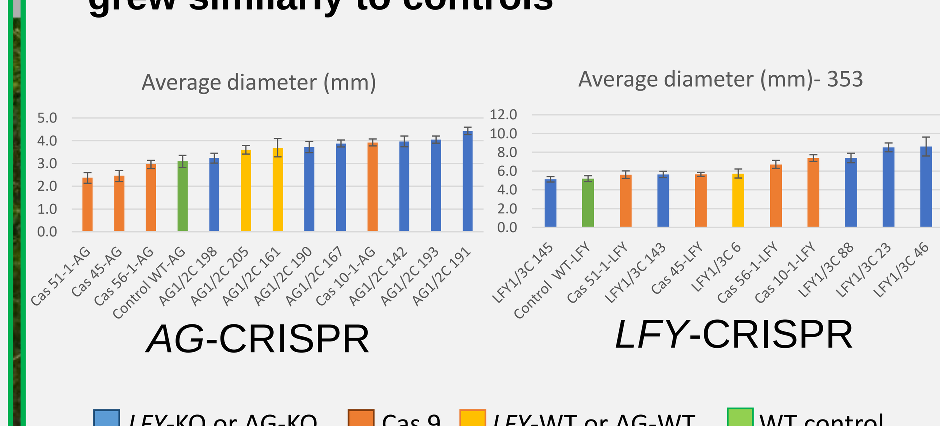


WT bud showing normal floral organs — **No floral organs seen in KOs-just vegetative layers**

Nearly all *Eucalyptus* SP7 *LFY*-CRISPR knockout events grew similarly to controls



All poplar AG-CRISPR & *LFY*-CRISPR knockout grew similarly to controls

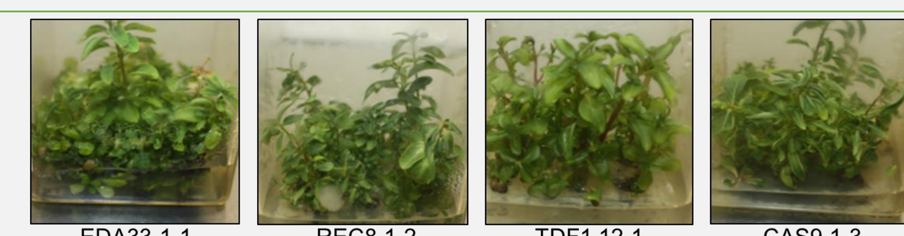


Three novel CRISPR constructs transformed into rapid flowering and wild type eucalypts

- Selected five genes should provide male, female or bisexual sterility in eucalypts
- Total of 48 events obtained from SP7 WT transformed with novel CRISPR constructs
- Preliminary studies show that we have confirmed KOs for *TDF1* and *EDA33*

Gene ID	Function	Predicted phenotype
AG	Stamen and carpel development	Bisexual sterility
EDA33	Seed pod valve development	Female sterility
LFY	Transition to flowering stage	Bisexual sterility
REC8	chromosome division in meiosis	Bisexual sterility
TDF1	Tapetal development	Male sterility

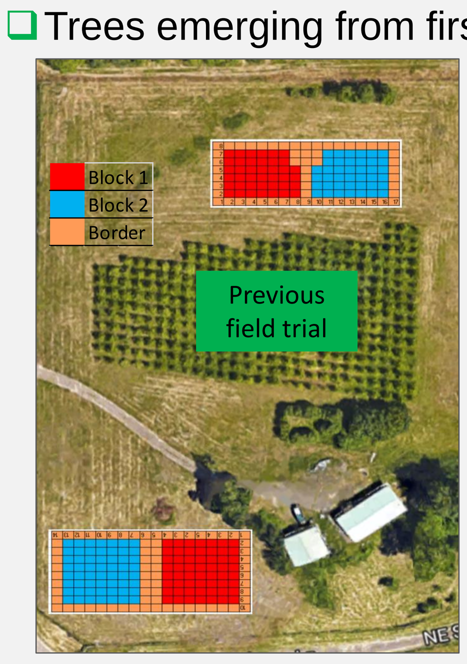
Sterility Gene (Hygro. selection)	Total number of explants transformed	Percent of explants formed callus	Percent of explants formed shoot	Total number of events obtained	Transformation rates (%)
EDA33	409	26.6	14.4	10	2.0
REC8	362	20.6	9.6	16	4.4
TDF1	428	39.8	18.2	15	3.5
CAS9	316	42.7	16.2	7	0.9



EDA33 1-1 — REC8 1-2 — TDF1 12-1 — CAS9 1-3

Gene editing field trials established

- Field trial of CRISPR poplar clones 717 & 353 targeting *LFY* and *AG* were established in Oregon in November 2017
- In total, 180 trees were planted in each of two blocks, plus a border block, for female clone 717; 136 trees were planted in two blocks plus border block for male clone 353
- Field events include:
  - Biallelic knockouts to test effects on flowering
  - Heterozygous events to test stability and allele conversion
  - Non-mutated CRISPR-transgenic events to test for activity over time
- Trees emerging from first dormancy this spring had nearly a 100% rate of survival



View of one block of field trial prior to weed control treatments in spring

SUMMARY

- CRISPR targeting 2 genes (*LFY* in eucalypts & *LFY* & *AG* in poplar) are undergoing greenhouse and field trials
- There was a very high knockout frequency in eucalypts (97%) and poplar (73%)
- CRISPR knockout trees are growing well in the greenhouse & largely show an absence of effects on vegetative growth and morphology
- Preliminary studies show that knockout eucalypts are sterile
- Transformed eucalypts with the 3 additional sterility gene targets are currently undergoing mutation analysis
- Field trials for transgenic poplar were planted in fall 2017

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TBGRC

USDA United States Department of Agriculture — National Institute of Food and Agriculture