**BACKGROUND**

- Field research and commercial use of transgenic plants are severely limited by regulations, and associated ecological and legal risks, for which transgene dispersal is major elements.
- These concerns warrant an efficient, reliable, and biosexually effective method for genetic containment of vegetatively propagated transgenic plants and other perennial crops.
- The Clustered Regularly Interspaced Short Palindromic Repeats (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 varieties.

**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 transgenic plants.
- Study methods of site-specific excision system of CRISPR/Cas9.

**APPROACH TAKEN**

- A targeted total of five genes that are essential to normal flowering (LFY, TDF1, AG, REC8, and CAS9).
- Re-transformed FT early flowering eucalypts with CRISPR to speed analysis of fertility.
- Performed greenhouse assessment to test flower (FT events) and gynoecium morphology.
- Established poplar field trial in Israel, and to establish a high yield field trial in Israel.
- Will study off-target mutagenesis in CRISPR transgenics using targeted knock-out methods.
- Will develop excision systems for removal of CRISPR locus using reverse genetics promoters.

**CRISPR transformation, mutation analysis, and phenotype pipeline**

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

<table>
<thead>
<tr>
<th>Gene</th>
<th>Total events</th>
<th>Mutation %</th>
<th>Frequency</th>
</tr>
</thead>
<tbody>
<tr>
<td>LFY</td>
<td>256</td>
<td>Barileic KO</td>
<td>105 (66%)</td>
</tr>
<tr>
<td>AG-CRISPR</td>
<td>74</td>
<td>WT</td>
<td>99 (34%)</td>
</tr>
<tr>
<td>AG-CRISPR</td>
<td>139</td>
<td>WT</td>
<td>99 (34%)</td>
</tr>
<tr>
<td>LFY</td>
<td>50</td>
<td>Barileic KO</td>
<td>50 (100%)</td>
</tr>
<tr>
<td>LFY</td>
<td>55</td>
<td>WT</td>
<td>50 (100%)</td>
</tr>
<tr>
<td>All poplar</td>
<td>480</td>
<td>Barileic KO</td>
<td>387 (80%)</td>
</tr>
</tbody>
</table>

**CRISPR eucalypts & poplars grow well in greenhouse experiments**

**Eucalyptus FT-LFY-CRISPR knockouts produce sterile and indeterminate floral buds**

- Nearly all Eucalyptus SPT LFY-CRISPR knockouts events grew similarly to controls.
- All poplar AG-CRISPR & LFY-CRISPR knockout events 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 eucalyptus.
- Total of 48 events obtained from SP7 WT transformed with novel CRISPR constructs.
- Preliminary studies show that we have confirmed KO's for TDF1 and EDAA3.

**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.

**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 eucalyptus (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 eucalyptus are sterile.
- Transformer eucalyptus 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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