Efficacy of RNAi and CRISPR Containment Technologies in Poplar

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Project Summary
The dispersal of transgenes from genetically engineered poplars presents substantial challenges to biotechnology regulatory bodies. This is because they are weakly domesticated, have wild relatives, and pollen or seeds can spread widely. However, plantation trees are vegetatively propagated, making fertile flowers unnecessary for commercial use. Thus, genes that induce complete sterility could provide strong and simple regulation of transgene spread. We have two study populations of transgenic poplar trees. The first is a field experiment used to test flat modification by RNA interference (RNAi) of floral development genes under natural flowering conditions. The second is a laboratory study of the efficacy and containment of floral genes using transgenic plants held in tissue culture for 2 years. We report on the progress of these studies and the outcomes of evaluations of containment methods. The first field test of genetic modification by RNAi of floral development genes is efficient and effective in male poplars. Overall, our work demonstrates that suppression of floral development genes in poplar is effective in male and female trees will be studied in the field in upcoming years, hopefully leading to a model to deploy containment technology.

Field Test of Floral Modification by RNAi of Floral Development Genes
We selected a variety of poplar genes from the field development pathway (see Table 1). We created 22 constructs targeting floral development genes, both singly and in combination. These were transformed into three poplar clones, one male and two females. We planted 4–25 independent transgenic explants per construct per clone. On average, we planted 4–7 trees (trees/sex) per event. Pairs of ramets were interplanted into two blocks per clone. Onset of sexual expression (stamens) was indicated by red and black flowers. Aerial view of the planted site. Trees are indicated by red and black lines.

CRISPR-Cas Targeting of LFY and AG Genes in Poplar
We are utilizing CRISPR-Cas mutagenesis to target the LFY and AG genes in poplar. This approach should lead to very strong and non-reversible changes to targeted genes. We predicted that deletion of this region will cause complete sterility by disrupting flower organ formation. To study this, we are targeting two regions of the LFY gene (partial gene deletion), either directly using CRISPR-Cas or with the help of an auxiliary Cas system (tracrRNA and crRNA) to enhance cutting efficiency. Construct trees with RNAi targeting LFY and/or AG were planted 2014. We have two study populations of transgenic poplar trees. The second is a laboratory study of the efficiency of direct modification by CRISPR Cas–associated Cas system (Cas9) for targeting of selected floral genes by Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR) technology. We predict that deletion of this region will lead to a complete lack of LFY function. We are targeting two regions of the LFY gene (partial gene deletion), either directly using CRISPR-Cas or with the help of an auxiliary Cas system (tracrRNA and crRNA) to enhance cutting efficiency. We created CRISPR constructs with one or two guide RNA sequences focused on our targets. A construct with just Cas9 serves as a negative control as we do not expect any DNA changes in the absence of the guide RNA.

All Poplar Clones Flowered Well in 2016 and Are Growing Well Across Constructs and Clones
The field plantation was established in 2011 as small rooted ramets; 40 clones were scored for flowering early; among the first flowers were observed in 2014. Examples of representative flowers for eight clones are shown below. In spring 2016 all three clones had flowering rates of 42.8–59.0%.

Table 1: Genes targeted for suppression or modified expression in transgenic poplar trees

<table>
<thead>
<tr>
<th>Gene</th>
<th>Targeting Type</th>
<th>Haplotypes</th>
<th>&lt;?= Table 1: Genes targeted for suppression or modified expression in transgenic poplar trees</th>
<th>Gene</th>
<th>Targeting Type</th>
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<tbody>
<tr>
<td>LFY</td>
<td>RNAi</td>
<td>X 717</td>
<td>X 353</td>
<td>X 6K10</td>
<td>RNAi</td>
<td>X 717</td>
<td>X 353</td>
<td>X 6K10</td>
<td>RNAi</td>
<td>X 717</td>
<td>X 353</td>
<td>X 6K10</td>
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<tr>
<td>AG</td>
<td>RNAi</td>
<td>X 717</td>
<td>X 353</td>
<td>X 6K10</td>
<td>RNAi</td>
<td>X 717</td>
<td>X 353</td>
<td>X 6K10</td>
<td>RNAi</td>
<td>X 717</td>
<td>X 353</td>
<td>X 6K10</td>
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<td>FPF1</td>
<td>RNAi</td>
<td>X 717</td>
<td>X 353</td>
<td>X 6K10</td>
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<td>X 6K10</td>
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<td>X 353</td>
<td>X 6K10</td>
<td></td>
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</tr>
</tbody>
</table>

RNL of LFY and/or AG Led to Reductions in Female Fertility
Seven of our BbBa constructs had trees with reduced floral morphology (clones are shown below). All of these constructs were designed to target LFY and/or AG, sometimes in combination with other floral genes. Constructs targeting just LFY led to trees with no open female flowers. Constructs targeting AG led to reduced numbers of carpels per flower. These female trees were scored as fertile. A new field study will be carried out in 2017 to assess the efficacy of these effects on reproductive growth. Pollen was collected in July 2016.

CRISPR-Cas Mutations Efficiently Targeted Poplar Floral Genes
Analysis of CRISPR events targeting LFY in clone 717 shows a high rate (12%) of viable, homozygous plants with mutations (indicated below). Of these 23%, 10% had mutations in both hemi-plants, and 13% had mutations in one-hemi plante. Of the events observed for changes in the LFY and/or AG genes, 60% had mutations, and 6% of events had alleles, homozygous mutations. These high rates of gene targeting means that CRISPR-Cas technology is a very efficient means for altering floral development genes in poplar. Overall, our work demonstrates that suppression of floral development genes is effective in male and female trees will be studied in the field in upcoming years, hopefully leading to a model to deploy containment technology.

Summary
- Trees for all constructs are growing well in our 9 x 9 experimental plantation, and nearly all constructs should have a majority of trees flowering in 2017.
- RNAi targeting of LFY and/or AG strongly decreases fertility in two female clones studied to date, and does not appear to affect vegetative growth or morphology.
- CRISPR-Cas modification of floral development genes is efficient and should lead to strong and stable disruption of gene function, and thus reliable genetic containment. A field test of growth rate and flowering is planned to begin in 2017.

CRISPR-Cas Mutations Detected by DNA Sequencing of Transgenic Plants

Microscopic analysis of control cuticles showed that the carpels contained well-formed ovules, which later developed into seeds. When ovaries were harvested just prior to dehiscence, few viable seeds were collected. The few viable seeds were harvested were impossible to retrieve in these carpels. Cuticles with targeted AG had carpels formed inside of other carpels, with them differing in height, a distinct replicated pattern. Suckering of these carpels showed that some events lacked ovules entirely, this will be fully sterile.

CRISPR single and double gene targeting constructs

C11 - 14-clonal population
C15 - 14-clonal population
C16 - 14-clonal population
C17 - 14-clonal population

Cas9 (black shape) interacting with the matched DNA target and guide RNA sequences

LTF target sequences:
-DACACAGCGCTAAAGGAGGTGCAGCTTGAACGTTGTGCACAACGTTATGGTGTTG
-GAGGGAAACAGACGTTCTTTGCCTGTGTCGAGCTGAGGCTG

Select Events for Rapid Flowering and Field Studies

 buzzy locus

BZZ1 locus

heterozygous

bi-allelic

allelic

homozygous

CRISPR-Cas Mutations Efficiendly Targeted Poplar Floral Genes

Table 2: Results of CRISPR targeting of LFY in clone 717

<table>
<thead>
<tr>
<th>Construct/Target</th>
<th>Sequence</th>
<th>Mutations (%)</th>
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<tbody>
<tr>
<td>Single LFY</td>
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<tr>
<td>Single LFY</td>
<td>20%</td>
<td></td>
</tr>
<tr>
<td>Single LFY</td>
<td>50%</td>
<td></td>
</tr>
<tr>
<td>Single AG</td>
<td>50%</td>
<td></td>
</tr>
<tr>
<td>Single AG</td>
<td>20%</td>
<td></td>
</tr>
<tr>
<td>Single AG</td>
<td>10%</td>
<td></td>
</tr>
<tr>
<td>Single AG</td>
<td>0%</td>
<td></td>
</tr>
<tr>
<td>Single AG</td>
<td>5%</td>
<td></td>
</tr>
</tbody>
</table>

Examples of identical, homologous mutations found in independent events targeted with a single guide RNA in LFY. Details indicate deleted bases, numbers on the right indicate how many events had a given mutation. Large mutations (not shown) were observed in events transformed with the double guide RNA construct.