Project Narrative
CRISPR-Cas9 mutagenesis for genetic containment of forest trees

Introduction
The undesired release of genetic material to the environment from transgenic plants presents a major ecological concern and regulatory barrier to their use (Tiedje et al. 1989, Rogers and Parkes 1995, Snow and Palma 1997, Auer 2008). Although transgenic trees may provide a valuable resource for wood production, biofuels, bioremediation, and many other applications (Groover 2007, Harfouche et al. 2011), commercial uses are severely limited by regulations and associated ecological and legal risks that are largely the result of transgene dispersal (Slavov et al. 2004, Williams and Davis 2005, Hoenicka and Fladung 2006, Chandler and Dunwell 2008, Kuparinen and Schurr 2008, Strauss et al. 2010 and 2015). The extensive geographic range over which pollen from trees such as poplars can disperse, often well beyond 10 km, makes gene dispersal especially problematic (e.g., Slavov et al. 2009, DiFazio et al. 2012). Similar issues exist for many ornamentals and grasses (e.g., Zapiola et al. 2008), including those intended for use as biofuels such as switchgrass (Stewart 2007, Strauss et al. 2010). Efficient, reliable, and bisexually effective methods for genetic containment may be essential to the broad use of vegetatively propagated transgenic trees and other perennial biofuels crops (Slavov et al. 2004, Brunner et al. 2007, Moon et al. 2010, Strauss et al. 2010, Vining et al. 2012).

A contemporary example of the pressing need for improved containment technology in trees to facilitate regulatory decisions is shown by the deliberations pertaining to cold-tolerant transgenic eucalypts (Voosen 2010, The Forestry Source 2010). These trees are being used in broad uncontained (i.e., flowering) field trials and an application for commercial release in the southern United States has been submitted, for which APHIS is producing an environmental impact statement (APHIS 2013). Concerns over release have been somewhat reduced by employment of strong pollen sterility technology in these trees (Strauss et al. 2011, Zhang et al. 2012); however, its efficacy may be limited as the technology is not expected to be fool-proof during large-scale use based on results using similar technology in field crops (e.g., temperature sensitivity of male sterility as seen in Brassica with barnase: Denis et al. 1993). In addition, even if male sterility holds, long-distance pollen movement from other planted eucalypts and the lack of female sterility is likely to result in seed development. The need for improved containment technology was supported by a survey of forest biotechnologists in the USA, which identified research on containment technology as the highest priority for application-oriented research in transgenic biotechnology (Strauss et al. 2009).

Containment technology does not need to be perfect to provide high levels of mitigation. Because vegetative spread in poplars is slow, mostly local, and easily managed, simulations of sexual sterility in poplars have shown that even modestly effective sterility technology could have a very large benefit for containment (DiFazio et al. 2012). Modest containment was also effective at restricting the spread of linked genes that provide a strong selective advantage. Thus, sterility genes—particularly the highly effective and putatively stable knock-out mutations from gene editing that we will test—should provide extremely high levels of mitigation.

Approaches to engineering plant sterility
A number of transgenic approaches are being studied for gene containment in forest and other plant species, including organ ablation, transgene excision, RNA interference (RNAi), transgene-encoded protein interference, and expression of genes that cause delays in onset of
flowering (reviewed by Brunner et al. 2007, Hüsken et al. 2010, Moon et al. 2010, Harfouche et al. 2011, Vining et al. 2012). To date only pollen sterility, using ablation methods, have been shown to successfully cause male sterility for trees under field conditions. However, there are only limited data on the stability of sterility transgenes in trees over multiple years, and only for pollen ablation methods (Strauss et al. 2011, Zhang et al. 2012, Elorriaga et al. 2014).

Stability in expression of a mitigation transgene is critical to its regulatory approval and commercial use (Ahuja 2009, 2011). Technologies that do not depend on gene expression—which can be modified by environment, development, and epigenetics—would be preferable for regulatory assurance of stability. A major advantage of direct gene editing over alternative technologies is the capability to produce permanent structural changes in gene sequences that disable their function. These changes can include the deletion of essential domains or entire parts of genes (as shown under preliminary studies), and can be identified in juvenile tissues years before floral onset. The loss of both copies of essential parts of a unique gene is unlikely to undergo a repair or reversion. Thus, genetic containment by gene editing should produce stable, predictable, and reliable forms of containment, facilitating regulatory decisions.

**Floral genes targeted for modification**

Our work to date in poplar and eucalypts has primarily focused on two well-known regulators of floral development whose mutation generally leads to complete or nearly complete bisexual sterility: LEAFY (LFY) and AGAMOUS (AG). We have recently created three new gene editing constructs for eucalypts that target additional classes of genes, and should be able to impart male, female, or bisexual sterility.

**LFY.** Flowers are formed from floral meristems that arise on the flanks of the main shoot apical meristems (SAMs). Their identity as reproductive tissues is conferred by floral meristem identity genes such as LFY which encodes a transcription factor (Weigel et al. 1992) that directs the expression of several classes of floral homeotic genes in overlapping spatial domains (Wagner et al. 1999, William et al. 2004, Denay et al. 2017). Strong homozygous LFY mutants largely produce vegetative shoots in place of flowers (Schultz and Haughn 1991, Weigel et al. 1992). Homozygous mutants of the LFY ortholog FLORIAUML derived from snapdragon (Coen et al. 1990) appear to be completely sterile, as are homozygous mutants for the tomato LFY ortholog FALSIFLORA (Molinero-Rosales et al. 1999). LFY is a single-copy gene in the large majority of dicotyledonous plants studied, including eucalypts (EgLFY: Southerton et al. 1998) and poplar (PtLFY) both of which show some vegetative expression (Rottmann et al. 2000). We have observed completely sterile flowers and normal growth in highly LFY-suppressed poplar in field trials, which we recently published in Nature Biotechnology (Klocko et al. 2016a, Fig. 1C).

**AG.** The floral homeotic genes, which predominantly encode MADS domain transcription factors (Krizek and Fletcher 2005), specify the identity of the sepal, petal, stamen, and carpel organs according to the well-characterized ABC model (Coen and Meyerowitz 1991) and its further elaborations. AG specifies the identity of the male (stamen) and female (carpel) reproductive organs (Bowman et al. 1989), and the loss of AG function generally leads to a complete or nearly complete loss of stamen and carpel development, and thus complete sterility. Poplar contains two very similar and recently duplicated genes with strong similarity to AG in cellular expression and amino acid sequence, including, however, some vegetative expression (the PtAGs: Brunner et al. 2000). The high DNA sequence conservation between the two PtAG genes enabled us to design our gene editing constructs to target highly similar domains in both genes with the expectation that both would be mutated under the high rates of CRISPR-Cas
mutagenesis, as indeed we have observed (preliminary studies). We have also documented sterile flowers and normal development in highly AG-suppressed RNAi poplars in field trials, supporting the suitability of AG as a target for genetic containment (Fig. 1B).

Novel eucalypt gene targets. As discussed under preliminary studies, based on a combination of eucalypt transcriptome analysis, bioinformatics, and review of Arabidopsis literature, we selected three genes for mutagenesis that are intended to give a variety of male, female, and bisexual sterility traits. The genes targeted include Eucalyptus orthologs of TAPETAL DEVELOPMENT AND FUNCTION 1 (TDF1). In Arabidopsis, this transcriptional factor is required for development of the tapetum and male fertility (Zhu et al. 2008) and functions as a positive transcriptional regulator of tapetal identity genes (Gu et al. 2014).

SYNAPTIC 1/REC8 (REC8) is a gene required for meiosis in males and females (Cai et al. 2003, Zhang et al. 2006). The encoded basal cohesin protein is highly conserved across kingdoms as part of a cohesin complex that connects sister chromatids and must be cleaved for chromatid separation and anaphase (Kitajima et al. 2003).

EMBRYO DEVELOPMENT ARREST 33 (EDA33) is a gene required for valve margin development and seed release (Liljegren et al. 2004). The encoded transcription factor appears to dimerize with several others and acts as a regulator of many genes, including of embryonic identity (Kay et al. 2013).

CRISPR-Cas gene editing

The clustered regularly interspaced short palindromic repeats (CRISPR)-Cas gene editing system—as adapted for use in plant genetic engineering—is the newest and most promising tool for site-directed modification available. The CRISPR-Cas system results in changes to gene targets through the actions of guide RNAs, which physically pair with the DNA target sequence and the Cas9 nuclease protein, creating double strand breaks at the target site. Repair of these breaks can lead to permanent alterations in the target sequence. Because of its ease of use and efficiency, it is already having major impacts on biological science and agricultural research (Belhaj et al. 2015, Montenegro 2016, Quétier 2016, Song et al. 2016). Its high efficiency makes...
it feasible to generate loss-of-functions mutants in one or more genes with a modest transformation effort (Bortesi et al. 2016, Cao et al. 2016, Ding et al. 2016).

Delivery of the CRISPR-Cas9 reagents for gene editing can be accomplished by a variety of methods (Komor et al. 2017, Luo et al. 2016, Schaeffer and Nakata 2016, Xu 2015). These includes transient transformation with plasmid vectors (bacteria, yeast, mammalian cells, or plant protoplasts), viral transformation, microinjection of RNA or ribonucleotide protein complexes, Agrobacterium-infiltration (plant tissues), or stable transformation of multicellular tissues (Agrobacterium or particle bombardment in plants). The most successful plant systems employ stable transformation followed by sexual reproduction to segregate the CRISPR-Cas genes away from the desired changes of interest. However, this approach is extremely time consuming or impractical in species with long juvenile phases, such as trees or clonally propagated plants (e.g., potatoes, fruit trees, vines, and many ornamentals), and extremely difficult or impossible in the complete sexually sterile trees we seek to produce. Thus we plan to adapt and test a recombinase-based excision system to enable CRISPR-Cas genes to be somatically removed from the genome following mutagenesis and plant regeneration.

**Off-target mutagenesis**

**CRISPR-Cas**: Apart from cancer cell lines, off-target mutagenesis by CRISPR-Cas nucleases appears to be low, though its rate is highly variable among studies (Bortesi et al. 2016). Nine studies that included A. thaliana, N. benthamiana, hybrid poplar, rice, soybean, sweet orange, and wheat found no mutations in off-target sites with up to 7 mismatches (Lawrenson et al. 2015, Sauer et al. 2016, Schiml and Puchta 2016, Wolt et al. 2016). However, other studies did detect low, but significant, off-target mutagenesis (Jacobs et al. 2015, Lawrenson et al. 2015, Xie and Yang 2013, Upadhyay et al. 2013, Zhang et al. 2014, Xu et al. 2015, Sauer et al. 2016). Xu et al. (2015) found mutations in T1 rice plants carrying both the Cas9 and sgRNA transgenes, suggesting that continuous presence of Cas9 protein in the cell increases the likelihood of off-target cleavage. Based on these studies, it is difficult to predict what might occur with continuous CRISPR-Cas expression in a tree over several years, as we plan to study.

We are aware of only three genome-scale studies of CRISPR off-target mutagenesis in plants (Feng et al. 2013, Zhang et al. 2014, Peterson et al. 2016). Feng et al. (2013) generated whole genome sequence with 60X coverage from five Arabidopsis lines and used Blastn to generate two lists of putative off-target sites. Though many dozens of sites were likely considered (no counts were provided), no new SNPs or indels were found. Zhang et al. (2014) generated whole genome sequence for seven T0 and two T1 CRISPR-Cas transgenic rice lines and interrogated over 70,000 target-related sites, but reported no new mutations. Peterson et al. (2016) used genomic sequences from pooled Arabidopsis leaf tissues to investigate off-target mutagenesis at 14 target sites. They searched for mutations in 178 off-target sites that had either 3 or 4 mismatches but found no evidence of mutation. However, because of their pooling method the per site/per line sequencing depth was too low to detect infrequent mutations. Our proposed genome-scale study of off-target mutations should add significantly to this young field of study.

**Cre**: Off-target mutagenesis from the Cre recombinase has received little attention. Mammalian cell-lines studies have detected recombinant Cre activity at cryptic loxp sites in vitro (de Alboran et al. 2001, Loonstra et al. 2001, Thyagarajan et al. 2000) and in vivo (Higashi et al. 2009, Schmidt et al. 2000). E. coli clones with mammalian genomic inserts that include cryptic loxp sites had defective growth when the Cre recombinase was expressed (Semprini et al. 2007). We are not aware of any off-target studies due to Cre activity in plants, however, when
Cre expression is continuous it is known to have a damaging effect on plant growth, leaf morphology, and flowering in several species (Coppoolse et al. 2003), suggesting that off-target impacts are significant. Our genome-scale study of Cre off-target effects is likely to be the first of its kind in plants.

**Site-specific excision systems**

Gene excision systems have been widely applied for removal of transgenic elements in animals and plants, often for the removal of a selectable marker after transformation to improve regulatory or consumer acceptance (Rukavtsova et al. 2013, Wang et al. 2010). Recombinases have been the most widely employed tools for gene excision. They were tested in plants shortly after their initial demonstration in mammalian eukaryotic cells, starting with the Cre/lox system (Sauer 1987), which is effective in a variety of dicot and monocot plant species (Fladung et al. 2005, Gidoni et al. 2008, Mészáros et al. 2014). We are unaware of any studies of excision systems in eucalypts, but three recombinase systems, R/RS, Cre/lox, and FLP/FRT, have been found to be active in poplar (Matsunaga et al. 2002, Fladung et al. 2005 and 2010, Fladung and Becker 2010). The efficiencies of excision ranged widely depending on method of induction (coexpression, chemical, or heat) and means of assessment (gene loss or cassette exchange). In all cases where comparisons were possible, Cre/lox worked as well or better than other systems.

A number of promoters active in different stages of plant development have been used to control gene excision via the Cre recombinase (reviewed in Kopertekh and Schiemann 2012). This includes germline, embryo, microspore, flower, and seed. The high efficiency of such promoters in diverse species—which have included tobacco, Arabidopsis, soy, rice, and Brassica—has made this excision approach nearly universal. We plan to use a WUSCHEL (WUS) promoter to drive a WUS-Cre protein fusion to enable programmed excision through the central cells of the shoot apical meristem (SAM). As standard poplar transformation and regeneration is based on callus formation followed by shoot differentiation, using a SAM promoter-based excision system should allow for transgene excision in developing shoots.

The promoter from WUS has been shown to promote expression at high levels in the organizing center (OC) of regenerating shoots (Yadav et al. 2011), but not in callus (Che et al. 2006). WUS is diffused from the OC of the shoot apical meristem (SAM) into the stem cell niche (SCN) as demonstrated in Arabidopsis by anti-WUS immunoblotting and with translational fusions of fluorescent reporters with Arabidopsis WUS (Yadav et al. 2011). Because fusion of a small reporter protein to WUS does not prevent diffusion from the OC to the SAM (Daum et al. 2014, Yadav et al. 2011, Zhang et al. 2017), we plan use a WUS-GFP reporter to monitor expression and diffusion, and plan to employ a WUS-Cre fusion protein to induce excision during shoot development.

**Rationale and significance**

**Project alignment with program goals**

This project will study the effectiveness of CRISPR-Cas9 mutagenesis to promote transgene containment. The project therefore satisfies many of the criteria of Program Area 1: Management Practices to Minimize Environmental Risk of GE Organisms. In particular, it addresses area 1c: “Development or evaluation of effective bio-confinement strategies, including molecular and/or genetic techniques, to limit gene transfer (gene flow) or outcrossing to sexually compatible organisms.” Our research also addresses priority 5d: “Research addressing off-target genotypic
and/or phenotypic effects in GE organisms developed using genome editing technology.”

**Regulatory value**

Regulatory agencies, including the USDA, EPA (where a pest-tolerance trait is involved), and the FDA (where a GE animal is involved), must consider the broad environmental effects of introduced transgenes that will spread and may persist in the environment. This evaluation is difficult at the research stage, but is extraordinarily difficult at the commercialization stage where there may be extensive movement and interaction with wild and managed environments. The wide potential spread, the propensity for establishment in weakly domesticated species, the irreversibility of wide dispersal, and the myriad of possible non-target organism effects, makes ecological risk assessment extremely difficult and socially contentious (Strauss et al. 2016a). Performing the needed research to satisfy regulators and avoid litigation—or to develop sufficiently high quality information to support environmental impact statements (as has been required, for example, in transgenic alfalfa, bentgrass, eucalypts, cotton, and salmon)—imposes very large costs and long delays on applicants and regulators alike. If sexual dispersal can be reliably prevented, the scope of required risk assessment would be greatly reduced. Instead, vegetative spread, which can be much more easily controlled and monitored for plantation tree species and environments (DiFazio et al. 2012), would become the main dispersal risk, greatly simplifying regulatory analysis. The immediate relevance to regulatory decision making of genetic containment technology was discussed above with respect to transgenic eucalypts in the USA. A reliable, gene editing-based containment technology, such as we propose to develop, would greatly simplify the current regulatory assessment for this and similar cases. If successful for poplar and/or eucalypts, these approaches could be applied to other forest trees and vegetatively propagated, non-fruit crop species to facilitate regulatory compliance and improve social acceptability.

**Economic benefits of transgenic trees**

If successful in reducing regulatory delays and roadblocks, the methods we develop will expedite the introduction of the significant economic and environmental benefits expected from transgenic trees. These include, for example, high productivity, improved biotic and abiotic stress tolerance, reduced or improved use of pesticides and herbicides, reduced energy and chemical use in paper and pulp production, and improved product quality and energy production from biomass (reviews in Lucier et al. 2004, Harfouche et al. 2011). It may also enable applications, such as the use of novel pest resistance genes or bioindustrial products, for which it would be extremely costly to gather the necessary non-target toxicology and ecological information for deployment of non-sterile trees.

**Science and biotechnology**

Science: Complete loss-of-function by mutagenesis of the LFY and AG genes in poplar and/or eucalypts should identify the specific organismal and developmental functions of these genes during vegetative and floral development for the first time. In addition to floral phenotypes, we may detect novel vegetative phenotypes, as vegetative functions have been increasingly identified for various floral developmental genes (e.g., Pabon-Mora et al. 2012, Posé et al. 2012). RNAi-LFY transgenic poplars in field trials have shown sterility as desired and a lack of significant vegetative impacts to date (Klocko et al. 2016a), suggesting that its disruption is a good gene target for bisexual sterility. However, qPCR studies have shown that
RNAi has not given complete loss of target gene expression, and the vegetative analysis was based on a small number of trees. Expanded, loss-of-function studies as we propose will help to clarify the function of the LFY gene in poplar, and as discussed below also in eucalypts.

**Use of poplar and eucalypts:** We will conduct a major part of this project in poplar (genus *Populus*) because it is important as a model species for tree molecular biology and biotechnology. In addition, it has significant commercial value for wood, energy, and ecological restoration (reviews in Bradshaw et al. 2000, Tuskan et al. 2006, Brunner et al. 2007, Jansson and Douglas 2007). It is therefore a logical choice for primary studies of new biosafety mechanisms for trees and other perennial crops. Well-established and efficient *Agrobacterium*-based protocols for transformation produce trees with highly stable transgene expression, very low levels of somaclonal variation, and a low insert copy number (Groover et al. 2004, Han et al. 1997, Tzfira et al. 1997a and 1997b, Li et al. 2008a and 2008b). Poplars are planted widely in many parts of the world, and transgenic poplars have been authorized for commercial production in China (Lida et al. 2004). The methods developed may therefore be of direct relevance for transgenic poplar biotechnology worldwide.

Eucalypts are the most widely planted hardwood (angiosperm) forest tree in the world (Myburg et al. 2014), and are often grown as an exotic where it can in some instances become invasive (e.g., Booth 2012). It’s use in the USA may grow, particularly with climate change and associated warming (Vance et al. 2014); however, it’s potential for spread and associated ecological impacts are major concerns for regulatory approval (Voosen 2010, APHIS 2013).

**Preliminary studies**

The proposed research is highly leveraged in that it builds on many years of prior research in our laboratory and that of collaborators. We have previously cloned the target AG and/or LFY genes from poplar and eucalypts, and using either qPCR or transcriptome data (Vining et al. 2015), studied their expression; made binary CRISPR-Cas vectors targeting single and multiple targets; transformed and regenerated poplars and eucalypts; and confirmed a high rate of mutagenesis from many of the constructs. Recently we have added produced three new CRISPR-Cas9 constructs that target eucalypt floral genes, and have begun to transform them.

**Regulatory permits:** We have obtained (or are in the process of obtaining) USDA APHIS permits for the proposed or relevant activities. These include our field trial of RNAi-transgenic poplars that has informed us about likely knock-out phenotypes (previous permit number 13-330-102r, renewal application number 17-012-101r) and CRISPR-Cas transgenic poplar outplantings planned for winter-spring 2018 (nearly finalized; application number 16-006-104r). Futuragene also obtained a permit from Israeli authorities that allowed them to
outplant RNAi eucalypts that were sent from our laboratory; their floral and vegetative phenotypes are currently under study, and they have tentatively agreed to host a field trial of gene edited eucalypts at no added cost (letter of collaboration).

**RNAi-induced sterility in field trial:** Prior studies have shown that knock-down of two of the major genes proposed for study, AG and LFY, give apparent sterility and normal vegetative development in poplar (Fig. 1). These transgenic trees were found after screening of a 9-acre, 4,000 tree field trial that was partly funded by a previous USDA BRAG proposal (e.g., Klocko et al. 2016a, Klocko et al. 2016b). These results suggest that the complete loss-of-function provided by CRISPR-Cas knock-outs of these genes will give desired phenotypes.

**CRISPR-Cas gives high rates of mutagenesis in eucalypt and poplar:** We have conducted pilot studies of mutagenesis in poplar and eucalypt transgenic plants derived from insertion of using single- and double-target vectors plus a Cas9-only control (Fig. 2). The results in female poplar (clone 717-1B4, *P. tremula x alba*) are most advanced, and have shown that mutation rates are high, with a knock-out rate of 50% and an absence of mutations in 34 Cas9-only gene insertion events (hereafter called “events”) (Table 1). Even higher knock-out rates, approximating 60%, have been seen after sequencing of several dozen insertion events each of poplar clone 353-53 (*P. tremula x tremuloides*, male) and eucalypt clone SP-7. As with 717, to date no mutations have been found after sequencing of nearly 30 Cas9-only insertion events in 353-53. A diversity of mutations were observed, most being small insertions or deletions; however, when two sgRNAs targeting different regions of the same gene were delivered, large deletions were also observed (Fig. 3).

**Early-floral induction of CRISPR knock-outs.** Both poplar and eucalypts have long juvenile periods. We have many years of experience in using overexpression of FT to obtain precocious flowering in both poplar and eucalypts. To assess the effects of knock-outs on floral morphology, we have re-transformed poplar knock-out events of LFY and AG (both loci) with the 35S:FT gene that is known to induce early flowering in poplar. We have also transformed an FT-based early flowering (Klocko et al. 2015) version of eucalypt clone SP-7 (*E. grandis x urophylla*) with three CRISPR-Cas constructs targeting eucalypt LFY parallel to those used for poplar (Fig. 2), and already have identified 20 knock-out insertion events. Using these transgenic plants we will be able to assess flowering

<table>
<thead>
<tr>
<th>Construct</th>
<th>Events sequenced</th>
<th>Type of mutation</th>
<th># of events (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Single LFY1</td>
<td>121</td>
<td>Knock-out</td>
<td>62 (51%)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Heterozygous or non-mutant</td>
<td>59 (49%)</td>
</tr>
<tr>
<td>Single LFY3</td>
<td>46</td>
<td>Knock-out</td>
<td>26 (57%)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Heterozygous or non-mutant</td>
<td>20 (43%)</td>
</tr>
<tr>
<td>Double LFY1-LFY3</td>
<td>89</td>
<td>Knock-out</td>
<td>44 (49%)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Heterozygous or non-mutant</td>
<td>45 (51%)</td>
</tr>
<tr>
<td>Single AG2</td>
<td>36</td>
<td>Knock-out</td>
<td>17 (47%)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Heterozygous or non-mutant</td>
<td>19 (53%)</td>
</tr>
<tr>
<td>Double AG1-AG2</td>
<td>123</td>
<td>Knock-out</td>
<td>59 (48%)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Heterozygous or non-mutant</td>
<td>64 (52%)</td>
</tr>
<tr>
<td>Cas (empty vector control)</td>
<td>34</td>
<td>Non-mutant</td>
<td>34 (100%)</td>
</tr>
<tr>
<td><strong>Total (w/out control)</strong></td>
<td><strong>415</strong></td>
<td><strong>Knock-out</strong></td>
<td><strong>208 (50%)</strong></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Heterozygous or non-mutant</td>
<td><strong>207 (50%)</strong></td>
</tr>
</tbody>
</table>

**Table 1: Summary of CRISPR mutants analyzed in female poplar 717-1B4.** Knockout events include those with two identical alleles (m1/m1) and those with a different mutation in each allele (m1/m2). Heterozygous (m/Wt) and no-mutation (Wt/Wt) individuals can be used for evaluation of further mutation and chimerism over time.
phenotypes in the greenhouse during the first year of the proposed research. The three newly created eucalypt CRISPR constructs are also now starting their transformation into these early-flowering eucalypts, and will be studied starting in the second year of the grant period.

**CRISPR-Cas transformed wild-type trees for field trials in eucalypt and poplar.** To enable field trials of vegetative growth and floral morphology/fertility in the absence of the pleiotropic effects on growth from FT overexpression (Klocko et al. 2015), we are transforming wild type eucalypt SP-7 and one additional clone of poplar. The eucalypt clone was transformed with a double-guide RNA version of LFY, and poplar was transformed with double-guide versions of LFY and AG CRISPR-Cas constructs. Both were also transformed with the Cas-9 only control construct ([Fig. 2](#)). For poplar, these are the same vectors that we used to transform early female clone 717-1B4 (*P. tremula x alba*), and are known to have high rates of gene targeting ([Table 1](#)). The poplar clones are the same ones used in our current RNAi field trial discussed above, thus we are confident that they grow well and start to flower after 3-4 growing seasons at our field site in Oregon. All of them should be fully regenerated, rooted, PCR confirmed, and analyzed for mutagenesis by fall 2017-winter 2018. To date we have transformed 1,676 explants and observed 813 regenerating shoots (29 PCR confirmed to date) in eucalypt SP-7 ([Fig. 4](#)), and transformed 2,247 explants and observed 835 regenerating shoots (825 PCR confirmed to date) in clone 353-53. As discussed above the transformations of non-early flowering, female 717-1B4 were already completed and screened for mutations ([Table 1](#)).

**Design of new eucalypt CRISPR constructs.** In addition to our work targeting the eucalypt LFY gene, we are interested in targeting genes that can give other floral phenotypes. To select genes, we first used our floral transcriptome database (Vining et al. 2015) to build a list of 87 floral-specific genes, and then ran BLASTp to find Arabidopsis homologs. We also scanned an annotated Arabidopsis transcriptome for meiosis-related keywords (Pina et al. 2005) and then searched for their eucalypt homologs. To assess functional conservation, we also studied the functional domains using Simple Modular Architecture Research Tool (SMART). Finally, we ran BLASTp against the *Eucalyptus grandis* genome and selected targets that do not appear to be functionally redundant with any paralogs. As discussed above, selected targets include Eucalyptus orthologs of *TDF1* required for development of the tapetum and male fertility (Zhu et al. 2008); *REC8*, required for cohesion of sister chromatids during meiosis in males and females;

---

**Figure 3:** Examples of CRISPR-Cas induced mutations in the poplar AG gene. Sequencing of confirmed CRISPR events showed that while some events had no changes in the targeted region of the AG gene (Wt transgenic), many events had deletions at one or both target sites. Sequences shown are from confirmed homozygous mutants. Large deletions, insertions, and inversions were also observed.
Figure 4: Transformation of early flowering and wild-type eucalypts with LFY CRISPR-Cas constructs. Explants from early flowering FT and wild-type eucalypts are undergoing transformation with CRISPR-Cas constructs targeting sites in the eucalypt LFY gene. Constructs with one guide RNA (EgLFY1, EgLFY2) or two guide RNAs (EgLFY1/EgLFY2) are being tested. Sequence analysis of mutation types and frequency is ongoing. (Right) an explant with numerous healthy shoots.

Experimental Plan

The overarching hypotheses that we will test are that CRISPR-Cas is an effective and stable means for producing loss-of-function mutations in selected floral genes, and gives rise to healthy and stably sterile trees with low rates of chimerism and off-target mutagenesis. We will also test a means for removal of CRISPR-Cas genes following mutagenesis. A summary of the main proposed research activities are given in Fig. 5 and 7.

Objectives and research methods

Greenhouse and field trials of CRISPR-Cas transgenic trees: The goals of this work are to assess the effects of loss-of-function mutations on vegetative and floral phenotypes, the stability of CRISPR-Cas caused mutations in the presence of continued CRISPR-Cas expression, the extent of chimerism, and the development of new target and off-target mutations. To accomplish these goals, in both the greenhouse and field we will include five classes of transgenic plants and controls: loss of function homozygous and heterozygous events, mutant/wild-type heterozygotes, CRISPR-Cas transgenic non-mutants, Cas9-only transgenic controls, and two types of wild type controls (from micropropagation and escapes from the transformation process).

Four types of populations will be studied (Fig. 5):
1. Early flowering eucalypts (greenhouse only). These events are in the FT-accelerated flowering background. Transformation is completed for LFY constructs and has begun for the three new CRISPR constructs (preliminary studies). The greenhouse study will contain 5 loss-of-function events each for LFY and the other target floral genes (TDF1, REC8, EDA33),
and at least 5 control events (or sources, for wild type) each of Cas9-only and wild type controls, with each type containing at least three randomly planted ramets each. The population will be analyzed for stem growth (diameter), height, plant morphology (e.g., leaf shape and size), and floral morphology and fertility (pollen viability and seed germination after cross-pollination).

2. Early flowering poplar (greenhouse only). We have used 35S:FT to re-transform 5 knock-out events of LFY and AG in both the female 717-1B4 and male 353-53 genotypes, as well as five Cas9-only events and controls as above. Twenty events will be grown and at least two flowering retransformed events per original knock-out event will be studied in depth. It will be analyzed similarly to the FT-eucalypt population above except that fertility itself will not be studied as early-flowering poplar generally does not produce viable pollen and seeds (Zhang et al. 2010). However, we expect major changes in floral morphology that will demonstrate an inability to reproduce (e.g., Fig. 1).

3. Natural flowering poplar (greenhouse then field trial in Oregon). As described under preliminary studies, wild-type clones 353-53 and 717-1B4 have been transformed with CRISPR-Cas constructs. For each target (two duplicate AG loci and the single LFY locus) in each of the male and female poplar clones, we will propagate and plant 5 knock-out events, two heterozygous events, two transgenic but not mutated events, five Cas9 control events, and 5 sources each of the two types of wild-type transgenic events described above. The goal of using the transgenic but non-knock-out events, and the mutant/wild type heterozygous events, is to study the rate of mutation and chimerism that might develop in trees during field growth. Trees will first be grown in the greenhouse and measured for height, diameter and overall vegetative features such as leaf morphology. They will then be assessed in the field for vegetative growth, floral and vegetative morphology, within- and among-tree chimerism in vegetative and floral characters, and CRISPR-Cas target and off-site sequence modification for at least two additional years through to flowering. Genome-scale target and off-site mutagenesis studies are described below. Floral assessments in the field grown poplars are likely to begin after the three-year grant period.

4. Natural flowering eucalypts (greenhouse then field trial in Israel). We used CRISPR-Cas9 to produce knock-out mutations of LFY in wild-type SP-7 eucalypts (preliminary studies), and are currently doing the same with the three new CRISPR constructs. We will propagate 5 events that are knock-outs at for each gene, plus 5 Cas9-only events and 5 wild type controls of each type, replicated and randomized as above. These will first be studied in the greenhouse at OSU for vegetative effects, then field tested in Israel similarly to the poplar field trial described above (letter of collaboration).
Greenhouse and field vegetative morphology: In addition to measurement of tree size (height, diameter) to determine growth rate, we will carefully survey all plants for variation in vegetative morphology as an indicator of pleiotropy; if variation is visually observed we will measure the phenotype in all trees within those constructs and controls. Traits measured could include leaf area, total chlorophyll content, petiole length, floral or vegetative phenology, leaf shape, and leaf specific mass (mass per leaf area). We will pay special attention to the effects of environmental stresses, such as hard freezes, windstorms, high temperatures, or pest damage.

Floral phenotypes: We will visually inspect all plants for evidence of floral buds and mature flowers, and quantify variation in floral morphology. Floral morphology will be analyzed by noting the presence/absence and number of expected floral whorls (as per Figs. 1). For our dioecious poplar clones, trees will be checked for either male or female fertility, while eucalypt trees will be assayed for both male and female fertility. Floral phenotypes and fertility will be assayed yearly in the field trials once flowering begins to assess phenotypic stability across growing seasons.

In poplar, we expect strong floral alterations in knock-out mutant trees, similar to those observed in RNAi trees (Fig. 1), while we predict that trees from heterozygous mutant/wild type events will have normal flowers. Heterozygous and transgenic non-mutant trees will be carefully monitored to determine if any branches of individual trees show different floral morphology from the rest of the ramets, which would be a strong indicator of continued CRISPR-Cas activity at the target gene. If such alterations are observed, we will sequence the target genes from samples collected from different branches of the same tree (discussed below). If the trees show stable genotypes and phenotypes over the years of study, it would indicate that it might be acceptable for CRISPR-Cas genes to remain in tree genomes, simplifying their creation and use. If we see instability, it would underline the need for excision methods, as we propose to develop.

Male fertility will be assessed by multiple means. Mature flowers will be screened for pollen grain presence/absence. Grains, if present, will be fixed for visualization of pollen grain morphology and for viability staining as per our previous work (Elorriaga et al. 2014, Klocko et al. 2015). If eucalypt pollen grains are viable, pollen performance will be assessed via controlled pollination experiments in which control flowers are hand-pollinated with test pollen. Pollinated flowers will be monitored for viable seed formation.

Female fertility will be initially checked by screening for normal ovule formation. Flowers will be hand dissected and visualized with a dissecting microscope (as per Fig. 1). A second screening will check seed formation and seed viability. In poplar field trials, we have found that there is sufficient natural pollen to support production of viable seeds, and the same is true of field grown eucalypts. The resulting capsules will be checked for seed formation and seed viability by germination in Petri dishes.

Finally, we will photograph the catkins and capsules in the field for future trait quantification (such as catkin size and color). For the highly modified events, we will fix the catkins and mature capsules in FAA for microscopic analysis. Additionally, we will snap freeze flowers for possible RNA extraction and future transcriptome analysis (beyond grant scope)—with the goal of determining how loss-of-function affects floral and vegetative development.

As the onset of flowering is often synchronous and thus can make collections from the large number of study trees difficult, in poplar we will also collect one to two branches that bear floral buds in spring just prior to bud flushing (they can be identified by the size and angle of their buds relative to the branch: Stanton and Villar 1996). This approach will allow the branches to be held in a cold room until ready for analysis, when they can be put in a laboratory that their
flowers can be analyzed at a convenient time and while fresh (directly after flushing). We used this approach to successfully identify the RNAi events with modified floral morphology in field-grown poplars as shown in Fig. 1.

All fixed flowers and those sampled directly after flushing will be analyzed microscopically using our Keyence microscope. We have been using it extensively for analysis of floral morphology (e.g., Fig. 1). It will enable floral morphology to be observed and quantified from the catkin to sub-organ scale in large numbers of samples.

Off-site and continued mutagenesis: We will examine the extent of off-target mutagenesis from CRISPR and Cre-loxp induced mutations from greenhouse and field environments. Further mutation and chimerism are possible given that the CRISPR-Cas locus is continuously present in our transgenic materials. Because of the wide range of timing of the different greenhouse and field experiments, we will collect tissue samples and extract DNA for long-term storage as the experiments are conducted, but conduct the genomic analysis during the last year of the grant period. As discussed below, we will use bait-capture sequencing to focus attention on the parts of the genome most likely to suffer mutations (with similarity to sgRNA and loxp targets).

However the large size of the baits (~120 bp), which extend well beyond the 23 or 34 bp sgRNA and loxp-targeted target sites, allow us to query a great deal of genomic DNA that does not closely resemble target sites for mutations. We plan for approximately 96 samples for study by sequencing, allocated to include a variety of poplar and eucalypt materials over a wide time frame. We plan for approximately 96 samples for study by sequencing, allocated to include a variety of poplar and eucalypt materials over a wide time frame. These will include:

1) Heterozygous (mutant/wild type) and CRISPR-Cas9 transgenic but non-mutant events, for evaluation of CRISPR on-target mutagenesis and chimerism over time.
2) CRISPR-Cas9 off-target mutagenesis and chimerism over time, including a wide variety of CRISPR-Cas9 expressing transgenic materials.
3) Cre-only and Cas9-only “control” events for testing mutagenesis in the absence of target sites (Cre) or targeting guides (Cas9).
4) Non-transgenic controls, including field sourced, micropropagated, and regenerated but non-transgenic “escapes.”

By study of non-CRISPR and non-Cre trees that have undergone organogenesis similar to that of the transgenic trees (“non-transgenic escapes”), we can assess the rate of somaclonal variation in our in vitro transformation systems free of the influence of actual transformation and Cas9 or Cre activity, and compare that rate to CRISPR-Cas and Cre effects.

The online bioinformatics tool Cas-OFFinder (Bae et al. 2014) will be used to locate putative off-target sites for all of our sgRNAs. During our preliminary searches, we have used this tool to locate sites with both PAM sequences recognized by Streptococcus pyogenes (NGG and NAG) with up to 5 mismatches, giving lists that ranged from 157 to 1,962 putative sites per sgRNA target. When searching for Cre off-targets, we will interrogate the poplar genomes for targets similar to the wild type loxp sequence and also for sequences matching the cryptic loxp sites identified in mammalian cells (Thyagarajan et al. 2000). We will search for putative cryptic loxp sites in 717-1B4 using the probe search option in the 717-1B4 genomic online database. A preliminary search found at least nine targets very similar to the wild type loxp sequence and twenty targets very similar to one of the cryptic sites.

The baits will be designed, and sequences mapped, to genomes chosen to most closely resemble that of our transgenic test genotypes. These include several aspen-type genomes for Populus tremula x tremuloides 353-53 (Populus tremula draft genome version 1.1, Populus
tremuloides draft genome version 1.1, and *Populus tremula x tremuloides* T89: Sjödin et al. 2009, Sundell et al. 2015; for *Populus tremula × alba* 717-1B4 we will use its own draft genome (Xue et al. 2015), and for *Eucalyptus grandis x urophylla* SP-7 we will use *Eucalyptus grandis* genome version 2.1.1 (Myburg et al. 2014). Because of the long baits (~120 bp), they should be effective across a variety of genotypes and related species. We will use biotinylated baits (such as MYbaits® from MYcroarray) and selectively sequence 20,000 loci, including baits for both loxp (poplar only) and sgRNA (poplar and eucalypt) putative off-target sites. Although our test genotypes are hybrids that are closely related, but non-identical, to the reference genomes, the high depth of sequencing of a limited number of loci will enable us to recognize and ignore SNPs and indels between the test genotypes and the references.

All libraries will be sequenced in one lane of Illumina’s HiSeq3000 platform to give expected 600X coverage. This high read coverage should allow us to find even low frequency, chimeric off-target mutations. The raw Illumina sequences generated will be deposited in the NCBI Sequence Read Archive after the removal of adapter sequences and poor quality reads using Trimmomatic (Bolger et al. 2014). Processed reads will be aligned to reference genomes using Bowtie2 (Langmead and Salzberg 2012). We will use the mutation caller software VarScan version 2.3.8 (Koboldt et al. 2012) to identify both SNPs and indel mutations. This software was highly accurate at identifying low frequency variants in sequencing data (Spencer et al. 2014). All detected mutations will be PCR amplified and resequenced for confirmation.

**Excision system for removal of gene editing machinery:** In this system we will first use a GFP marker to study promoter and recombinase activity, and then test its mutagenesis and excision rate with a CRISPR gene in place of the marker. Previous research has demonstrated the reliable excision during germline development of seed-produced, transgenic *pCLV3::Cre* Arabidopsis (Van Ex et al. 2009). However, transformants of eucalypts, poplar and many other plants are generated by organogenesis; therefore, the use of a similar system for these plants will require excision during in vitro regeneration. *pCLV3::GFP* expression is reported to occur only in the shoot apical meristem (SAM) boundary during somatic embryogenesis of Arabidopsis (Su et al. 2009). This raises concern over whether recombinase activity will occur throughout the SCN, and thus whether a limited domain of expression may lead to chimerism of regenerated plants. We will therefore use the *WUS* promoter, which, together with WUS protein fusions, should enable a broader zone of excision.

We propose to direct expression of the *Cre* recombinase genes throughout the SCN of the SAM during regeneration by utilizing translational fusion proteins of Cre with WUS. As discussed above, WUS is expressed in the OC of regenerating shoots but not in callus. WUS is diffused from the OC into the SCN, and fusion of a GFP reporter to WUS did not prevent diffusion, thus we anticipate a similar diffusion pattern for a WUS-Cre fusion protein (GFP and Cre are both small proteins, 27 and 38 kD, respectively). All of the experiments discussed below will be in *Populus tremula x alba* clone 717-1B4, the most easily transformed genotype in our laboratory.
An overview of the proposed research constructs is given in Fig. 6. We will first test the system using two lengths of WUS promoter driving a WUS-GFP fusion protein. Alignments of poplar and Arabidopsis WUS promoter regions shows that the promoter element (called response element 1, RE1) shown to be required for SAM-localized expression of WUS in Arabidopsis (Bäurle and Laux 2005) is not clearly recognizable in *P. trichocarpa* WUS1 or WUS2. However, WUS2 has been shown to be upregulated during *in vitro* shoot induction in poplar (Bao et al. 2009a). Bäurle and Laux (2005) recognized a meristem specific enhancer between -1,627 and -1,360 in Arabidopsis, and our preliminary studies have shown that this region aligns most closely to the region of *pWUS2* starting at -1,680; we will therefore include 1,680 bp of *pPtWUS2* as a “short promoter”. Bao et al. (2009b) studied transcriptional fusions of GUS driven by *pPtWUS1* with promoter length of 3,492bp, a length selected because it includes most elements evidenced to be positive REs by computational RE prediction using PLACE (Bao et al. 2009b). This 5’ terminus aligns most closely to -3,391 of *pPtWUS2*, thus we will generate an additional set of constructs including 3,391bp of *pWUS2* (the “long” promoter). We expect the two promoter lengths will give more specific vs. more generic expression patterns, with hopefully one providing the needed specificity for programmed, but not premature, excision during shoot development in most events.

Expression of translational fusion proteins of WUS with fluorescent reporters can rescue *wuschel* mutations in Arabidopsis when the fusion is at either the carboxy or amino terminus of WUS; migration patterns are consistent regardless of which terminus the fusion is positioned at (Yadav et al. 2011, Daum et al. 2014). We will clone constructs with GFP and with Cre fused to the 3’ end of the ORF of *P. trichocarpa* WUS2 (*PtWUS2*), which should leave encoded domains essential for translocation unaffected.

We will be able to visually and non-destructively track expression by use of the WUS-GFP fluorescent fusion protein, which should be clearly visible in developing shoot meristems under the GFP-filter in our laboratory dissecting scope (e.g., Lu et al. 2016). We routinely utilize GFP expression as an indicator of transgene expression and know that it is highly visible in developing poplar and eucalypt tissues. We will then characterize cellular patterns of GFP expression in the SAM of several insertion events in depth using confocal, fluorescence microscopy ([OSU Genomics Center](https://www.genomics.oregonstate.edu); service fee includes training).

---

**Figure 6: Recombinase constructs for CRISPR-Cas removal.** The recombinase test system consists of (A) two lengths of the WUS meristem promoter controlling expression of a WUS-GFP translational fusion, and subsequently a WUS-Cre fusion, (B) a GFP marker gene cloned between two *lox* sites to examine recombinase activity. Using primers 1 and 2 will verify presence of the DNA between the *lox* sites, primers 1 and 3 and GFP fluorescence can be used to verify DNA excision. (C) The final construct used for removal of the entire CRISPR cassette during shoot regeneration but after mutagenesis.
Once the system is characterized with the GFP reporter fusion, we will test recombinase excision using a 35S:GFP transgenic poplar flanked by loxP sites (Fig. 6). Transgenic plants with this construct will be retransformed with a WUS-Cre fusion driven by the longer and shorter WUS promoter. We will use a hygromycin selectable plant marker (hpt) to enable Cre retransformation using nptII as a selectable marker. The extent of excision will be assessed in at least 100 transgenic shoots using GFP as a marker. In this system, GFP will be visible in developing calli but should be absent in shoots, as WUS::WUS-Cre should lead to excision of the 35S:GFP cassette in the SAM. We will then also use PCR to examine excision (Fig. 6); as a more sensitive assay, it should be able to detect chimerism in regenerated shoots that do not show detectable GFP expression.

If we find satisfactory rates of excision, we will then test whether the system can provide both high rates of mutagenesis and excision. We will use a selected CRISPR-Cas cassette that has high activity (e.g., one of those shown under preliminary studies). We will also transform the CRISPR cassette in the absence of the Cre gene, but with the same plasmid otherwise, so that the change in rate of mutagenesis compared to stable CRISPR-Cas9 overexpression can be determined. For these experiments we will use plant codon-optimized Cre gene and loxP sites that were provided to us by Pioneer/DuPont, and that has been shown to be highly effective in plants.

A summary overview of the experimental plan is provided in Fig. 7.

**Figure 7: Overview of experimental plan.**

**Quantitative analysis:** All data will first be checked for outliers, and those corrected or removed if justified based on statistical and/or independent experimental information. Vegetative data is expected to be largely continuous, whereas floral data is expected to be largely non-continuous (e.g., loss of flowers or specific organs). After consultation with our departmental statistical consultant, we expect that most analyses of continuous variables will be implemented via R or the SAS GLM procedure; both platforms enable all sources of variance, including the
effects of constructs, mutations, blocks, and covariables to be jointly analyzed. We will also map continuous phenotypes in regards to tree location in the greenhouse and the field, and statistically correct growth data for major patterns of variation (e.g., as done in Klocko et al. 2016a). For parametric analyses, assumptions of normality will first be assessed via visual display of residuals and transformations of data made as needed. If non-normality persists, non-parametric methods will be pursued. For non-continuous and attribute data, we will use Chi-square, binomial, or Fisher’s Exact Tests. These will be used, for example, to compare the rate of mutagenesis among constructs, and the rate in the presence and absence of recombinase mediated excision.

Roles of key personnel

**Steve Strauss** is a professor and will serve as PI. He has worked in poplar and eucalypt biotechnology and genomics for two decades, and conducted numerous field trials with transgenic poplars (Strauss et al. 2016b). He will oversee the entire project, including personnel, budgeting, field management, data analysis, and reporting.

**Amy Klocko** is a postdoctoral associate and will be responsible for creation of the meristem reporter and recombinase constructs and related tissue collections, microscopy, and DNA extractions. She will play a major role in floral morphology and fertility analyses, bait-target sequencing, data analysis, and reporting.

**Kelly Vining** is an Assistant Professor with extensive experience in genomic sequencing and data analysis. She will be responsible for advising the postdoc in the creation of the sequencing libraries, bait-capture methods, and genomic data analysis.

**Cathleen Ma** is a Senior Research Assistant II, who has worked in poplar and eucalypt biotechnology for more than two decades. She will be responsible for all *in vitro* and propagation and greenhouse work, including transformation, determination of transgene presence, and management of associated student workers.

**Estefania Elorriaga** is a senior PhD student working on CRISPR-Cas mutagenesis for her thesis. She will be responsible for CRISPR mutation analysis and greenhouse studies of wild type and early flowering poplar (she is supported by University funds)

Potential pitfalls and limitations, and strategies to adapt to them

*Will the field trials successfully grow and survive?* We have seen very little stress-associated damage in the many years we have tested the same genotypes at the field sites in Oregon or Israel. This includes a lack of frost, heat, or insect/disease damage (in spite of some very high and low temperatures recorded in recent years). A similar but much larger (9 acre) field trial of RNAi trees at the same Oregon State University farm planned for our field trial had a survival rate of nearly 99% since planting in 2011 (described in Klocko et al. 2016a). Thus we are confident that the much smaller trial we have proposed will be successfully planted and managed. This site has a locked gate, 10 foot high deer fencing, and irrigation to help trees get established. A similar irrigated and fenced field site will be used in Israel.

*Will the field trials flower during the project period?* We do not expect field flowering to begin to a significant degree during the 3 year project. However, loss-of-function mutations will also be evaluated in early flowering genotypes in the greenhouse (to characterize floral morphology, and by inference possible fertility), thus we will know the floral/sterility
phenotypes during the first year of the project. Vegetative data will be taken each year regardless of flowering to study pleiotropic effects—a key goal of the field trials. Futuragene has successfully tested eucalypt clone SP-7 over many years at their field site, where it often flowers during the first year of growth, and heavily during the second year of growth.

*Will recombinase activity excise the CRISPR-Cas genes prior to mutagenesis?* By use of the developmental promoter, we believe that a large number of events will not excise prematurely. By first comparing the rate and timing of excision of two different lengths of *WUS* promoters with GFP reporters, we expect that we will identify one that has a useful timing and rate of excision when used for mutagenesis. If this approach fails, prior work in our laboratory has shown that a *WUS1* promoter gave meristem activity in the majority of transgenic lines (Bao et al. 2009b). If the *WUS2* promoter and translational fusions give too limited expression for full excision, we could test the *WUS1* promoter as a likely means to get strong and specific of recombinase expression. We may also choose to test one of the published systems for chemical-induced recombinase induction; the laboratory has worked with extensively with chemical induction systems (e.g., Filichkin et al. 2006, Dalton et al. 2011).

**Schedule of work**

Colors show seasons: Winter (blue), Spring (green), Summer (yellow), Fall (brown)

<table>
<thead>
<tr>
<th>Start = January 2018</th>
<th>Year 1</th>
<th>Year 2</th>
<th>Year 3</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Category of Work and Planned Activities</strong></td>
<td>Q1 Q2 Q3 Q4</td>
<td>Q1 Q2 Q3 Q4</td>
<td>Q1 Q2 Q3 Q4</td>
</tr>
<tr>
<td><strong>Phenotypic evaluation in greenhouse and field</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Poplar</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Study vegetative and floral morphology in greenhouse - accelerated flowering and wild-type genotypes</td>
<td>X X</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Establish field plantings of <em>LFY</em> and AG knock-out trees in normal flowering, wild type poplar</td>
<td>X X</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Vegetative growth and leaf morphology assessments - field</td>
<td>X X X</td>
<td>X X X</td>
<td>X X X</td>
</tr>
<tr>
<td>Floral morphology assessment in field (mostly post grant)</td>
<td></td>
<td></td>
<td>X</td>
</tr>
<tr>
<td><strong>Eucalyptus</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Study vegetative and floral morphology in greenhouse - accelerated flowering and wild-type <em>LFY</em> mutants</td>
<td>X X</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Three new CRISPR constructs, morphology and fertility assessment in greenhouse</td>
<td>X X</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Field study of growth and flowering in <em>LFY</em> and new CRISPR constructs in wild type genotype (Israel, collaborator)</td>
<td></td>
<td>X X X</td>
<td>X X X</td>
</tr>
<tr>
<td><strong>Chimerism and off-target mutagenesis</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Greenhouse: Take samples from poplar <em>LFY/AG</em> CRISPR transformants, Cre excision lines, and eucalypt <em>LFY</em> CRISPR</td>
<td>X X X</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Greenhouse: Take samples from eucalypt transformants with three new euc CRISPR constructs</td>
<td></td>
<td>X X X</td>
<td></td>
</tr>
<tr>
<td>Take samples from poplar CRISPR transformants in field</td>
<td>X X X</td>
<td>X X X</td>
<td>X X X</td>
</tr>
<tr>
<td>Take samples from eucalypt CRISPR transformants in field</td>
<td></td>
<td>X X X</td>
<td>X X X</td>
</tr>
<tr>
<td>Baits, sequencing, bioinformatics</td>
<td></td>
<td></td>
<td>X X</td>
</tr>
<tr>
<td><strong>CRISPR excision system</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Build, transform, and evaluate meristem-reporter constructs</td>
<td>X X X X X X</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Build and evaluate SAM directed test constructs</td>
<td></td>
<td>X X X X X</td>
<td></td>
</tr>
<tr>
<td>Test selected CRISPR/recombinase construct</td>
<td></td>
<td></td>
<td>X X X X</td>
</tr>
</tbody>
</table>