Design of a self-excising CRISPR/Cas9 editing system for asexually propagated plants

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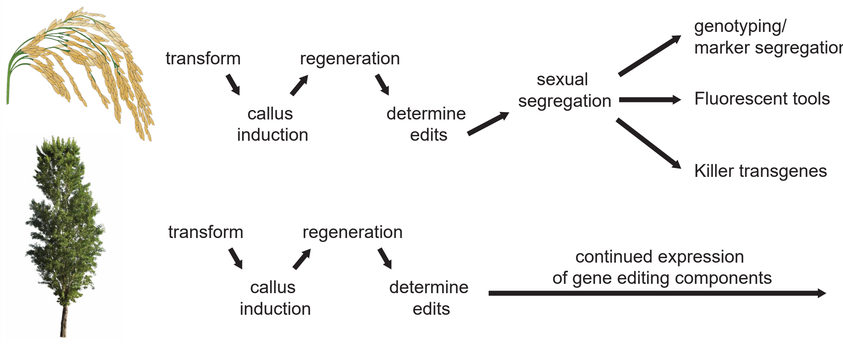
Introduction

Removal of gene editing components in plants: true breeding annuals vs. asexually propagated trees



Our primary interest lies in the development and use of genome editing tools to further the genetic improvement of forest trees. Many of the relevant lines we work with are interspecific hybrids of the *Populus* or *Eucalyptus* genera, which are asexually propagated. Typical delivery of sgRNAs and Cas9 editing materials in plants occurs through stable agrobacterium mediated transformation. Several methods have now been shown for improved elimination of transgene components in selfing plants, but few present systems have been adapted for use with asexually propagated plants. Routine gene editing performed so far in our laboraty generates trees with continued expression of sgRNAs and Cas9 throughout the life cycle.

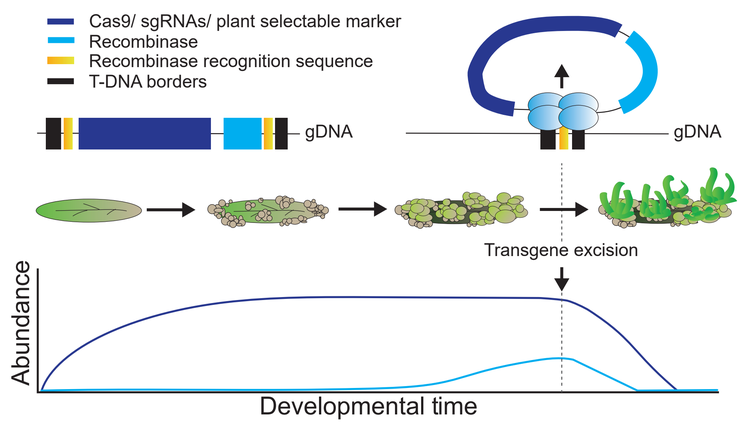
A developmental approach to CRISPR/Cas9 removal

How to perform somatic transgene removal of gene editing components?

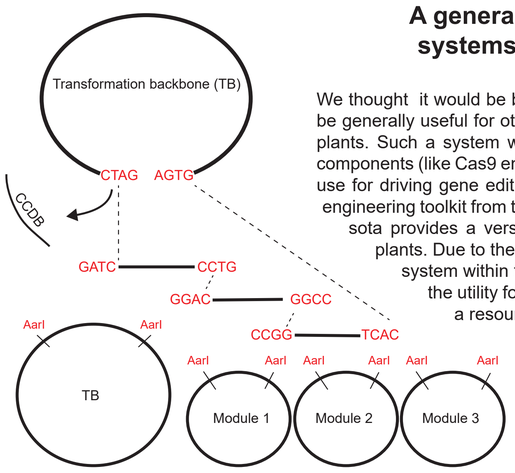
The use of site-specific recombinases for removal of transgenes has been developed and shown previously, and we reasoned that we could use this system to excise transgenes which contain the genes for sgRNAs and Cas9. If editing is to be performed, there needs to be enough time before excision is to take place and the transgene is eliminated.

We reasoned that temporal separation through developmental expression could be a valid approach. In short, sgRNAs and Cas9 could be expressed from contitutive promoters during transformation and callus outgrowth. During the shoot regeneration process, a recombinase could be expressed from a promoter localized only in the shoot apical meristem (SAM), to catalyze excision of the transgene. Transgene free cells in the meristem would further populate as the plant grows, and hopefully non-chimaeric plants could be obtained.

Schematic for transgene removal process



Adaptation of mature gene editing systems in plants



A generalizable tool for other systems as well as our own

We thought it would be best to design a system which could be generally useful for other models of asexually propagated plants. Such a system would be optimal if it had swapable components (like Cas9 enzyme), and a variety of promoters to use for driving gene editing components. The plant genome engineering toolkit from the Voytas lab at University of Minnesota provides a versatile framework for gene editing in plants. Due to these benefits, we sought to deisgn our system within this existing framework, to maximize the utility for our own group, and also to provide a resource for other groups.

From Čermák et al. 2017 *Plant Cell* PMID: 28522548

Construct design

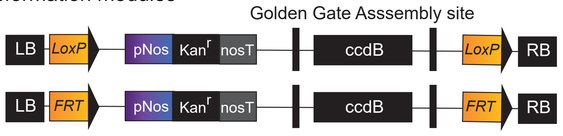
System design, modifications and implementation

Cas9 module

sgRNA modules

Recombinase modules

Transformation modules



eGFP-Cas9

Fluorescent Cas9 protein will allow for visualization of transient expression and zones of transgenic callus during outgrowth. It will also serve as a marker for mosaicism after gene excision is induced.

sgRNA arrays

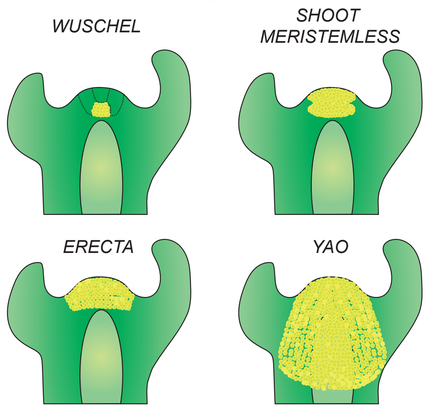
This vector will leverage existing modular sgRNA construction that allows tandem arrays of up to six guides expressed under a single promoter (tRNA or Ribozyme compatible).

"Lean" binary backbones with assembly sites and recombinase recognition sites

The binary backbone for final assembly of the gene editing and excision components is derived from the pCLEAN series of binary vectors. These are smaller, and have optimized T-DNA borders compared with some older vector systems. Golden gate assembly sites and a ccdB cassette within them enables high fidelity for obtaining a complete 3 module insert.

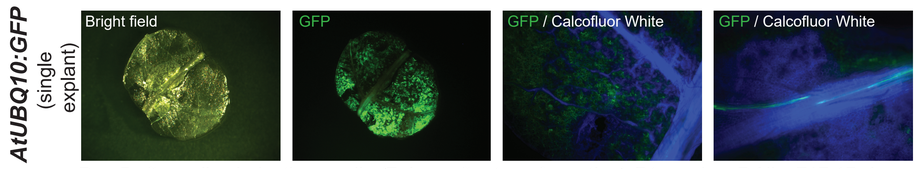
Shoot meristem promoter analysis during regeneration

Candiate promoters for further study during regeneration



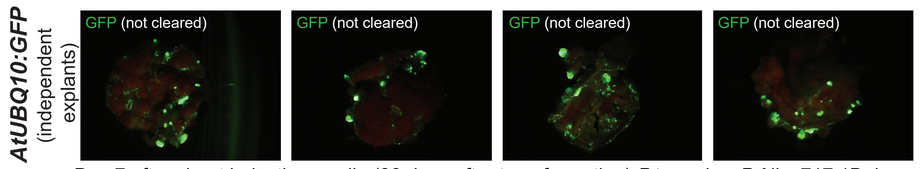
Many SAM promoters are well characterized in Arabidopsis or other species

Fluorescent protein compatible optical clearing methods should enable adequate visualization of emerged shoots



Day 5 after callus induction media (7 days after transformation) *P.tremula* x *P.Alba* 717 1B-4

Preliminary testing with a constitutive promoter suggests an optimal window to capture early shoot differentiation



Day 7 after shoot induction media (30 days after transformation) *P.tremula* x *P.Alba* 717 1B-4

Testing successful editing and recombination

Present Status

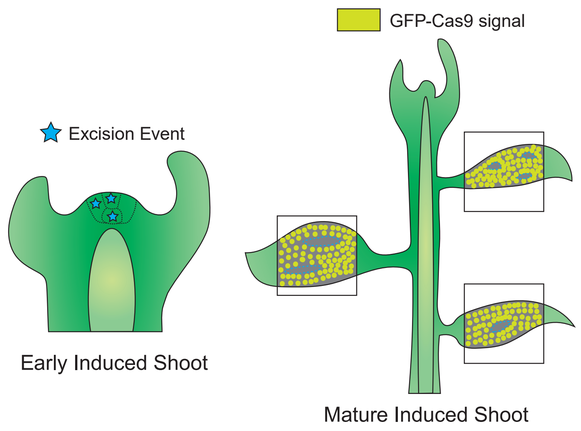
Vector cloning

Currently, we are in the process of making the transformation backbone vectors and assembling the recombinase modules.

Promoter Analysis

We have so far been using the *P.tremula* x *P.Alba* 717 1B-4 clone as source material for promoter expression analysis, as it is very robust to transformation. We are currently investigating *WUSCHEL*, and *YAO* expression (the most restricted and most general) and will soon be testing other promoters. In general we are using both Arabidopsis and *P. trichocarpa* promoters to test.

Detecting mosaicism in regenerated shoots



Primary optimization criteria

Shoot promoter choice

A critical mass of cells at the shoot apex where recombination has succeeded is required for transgene excision to be inherited in future cells at the SAM, and in newly emerged lateral organs. We anticipate that a promoter which expresses as broadly as possible within the meristematic central zone (CZ) will provide the best functionallity for the recombinase. If an adequate promoter is not found, tethering the recombinase to trafficing domains of WUSCHEL (WUS) or SHOOT MERISTEMLESS (STM) may provide mobility between cells in the shoot apex to improve function.

Recombinase protein and excision temperture

In this study we will test the function of the Cre and Flp recombinases (derived from P1 bacteriophage and *S. cerevisiae*, respectively). Their function has been investigated sparingly in plants, and usually expressed under heat-shock promoters. Cre likely functions better at temperatures outside normal regeneration growth conditions, and a mild temperature rise during excision may need to be necessary to improve recombination rates.

References

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Acknowledgements

This work is supported through the Biotechnology Risk Assesment Grant program (BRAG) no. 2010-33522-21736 from the USDA National Institute of Food and Agriculture. We thank Daniel Voytas from the University of Minnesota for vectors, Srinivasan Ramachandran from the Institute for Molecular Agrobiology in Singapore for promoter analysis vectors. and Michael Freitag of Oregon State University for site specific recombinases.