# MAR1 for Co-editing in *Populus* and *Eucalyptus*

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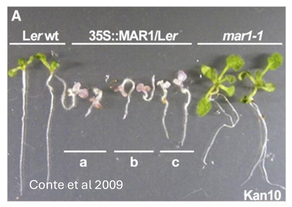
## Co-editing as a transgene-free transformation strategy

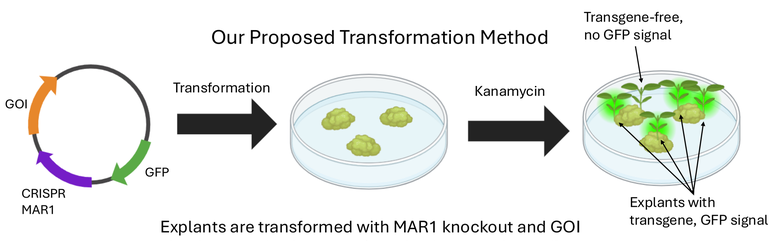
- Transgene-free transformation is desirable but difficult in clonally propagated crops
- Co-editing relies on transient expression of DNA to simultaneously introduce a selectable marker and desired edit without transgene integration
- Previous co-editing systems use a base editor to introduce a premature stop codon in the ACETOLACTATE SYNTHASE (ALS) gene (Hoengenaert et al 2025) but this limits Cas choice
- Knockout mutations in MULTIPLE ANTIBIOTIC RESISTANCE 1 (MAR1) confer aminoglycoside antibiotic resistance in *Arabidopsis* (Conte et al 2009) and could be used for co-editing
- We produced MAR1 knockouts to test MAR1 as a co-editing system

## MAR1 as an alternative selectable marker for co-editing

Kanamycin sensitivity of MAR1 mutants in Arabidopsis




Our Proposed Transformation Method




Explants are transformed with MAR1 knockout and GOI simultaneously, then transferred to kanamycin media to select for transgene-free edited plants (no GFP signal)

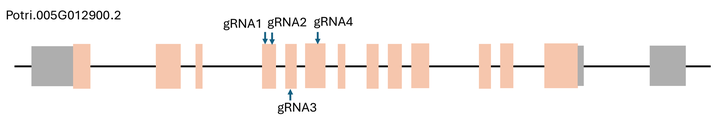
## Two co-editing constructs were designed


**CRISPR PtMAR1 PtLFY**

- All successfully transformed explants are antibiotic resistant and have LFY knockout
- Transiently transformed explants do not have GFP fluorescence


**AtMAR1 CRISPR PtMAR1 PtLFY**

- All successfully transformed explants are antibiotic resistant and have LFY knockout
- Transiently transformed explants do not have GFP fluorescence
- Explants with integrated T-DNA are sensitive to antibiotics




Poplar MAR1 gene with four gRNA targets in three different exons

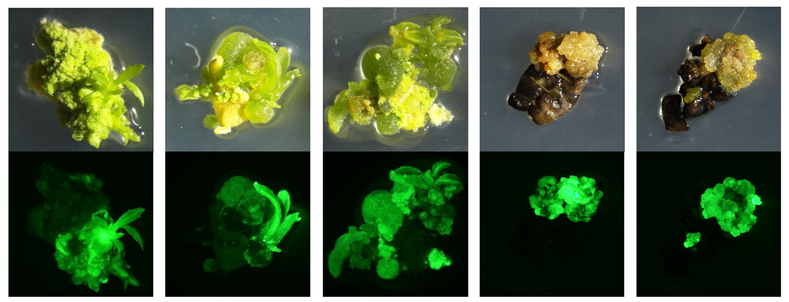
## Methods

1. Produced three different *Agrobacterium tumefaciens* clones, two with MAR1 knockouts and one empty vector control
2. Inoculated poplar tissue with *Agrobacterium* clones
3. Screened explants for GFP-positive, hygromycin resistant events
4. Regenerated plantlets in tissue culture
5. Sequenced events to screen for successful MAR1 knockouts
6. Tested kanamycin sensitivity of all knockout events with leaf discs on shoot inducing media

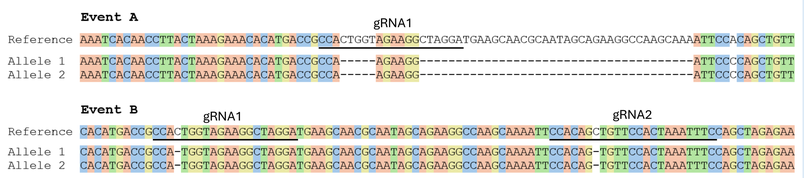
## Transformation with CRISPR MAR1 constructs produced multiple transgenic lines

- After selection, GFP screening produced 14 CRISPR PtMAR1 PtLFY and 24 CRISPR AtMAR1 PtMAR1 PtLFY transgenic poplars
  - The PtMAR1 knockout was successful in four of ten sequenced CRISPR PtMAR1PtLFY lines (40%) and eight of seventeen sequenced AtMAR1 CRISPR PtMAR1 PtLFY lines (47%)
- Editing with MAR1 knockout did not significantly impact transformation rate
- Transformants showed kanamycin insensitivity up to 25 mg/L, while WT control explants are kanamycin sensitive at this level
- In eucalypts, GFP callus was produced, but no transgenic shoots have yet been generated

## GFP positive events in poplar and eucalyptus

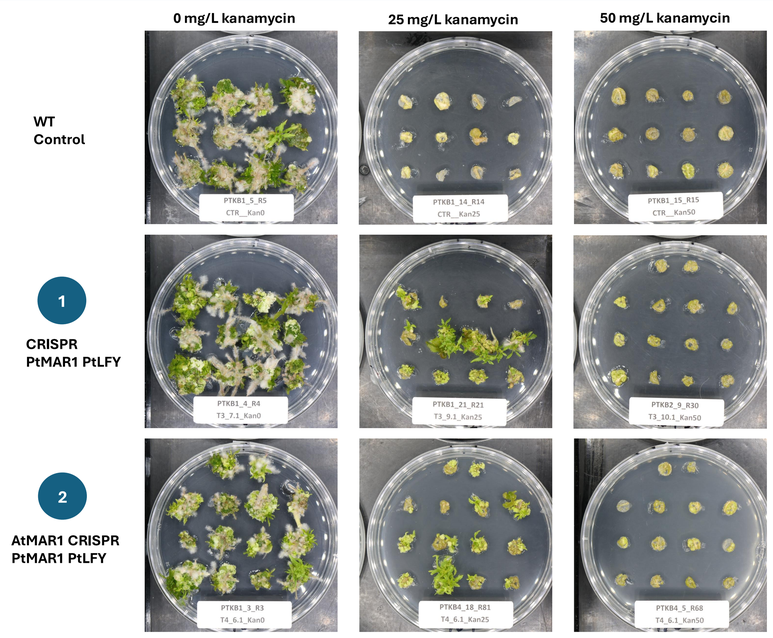



## Sequencing identified knockout events

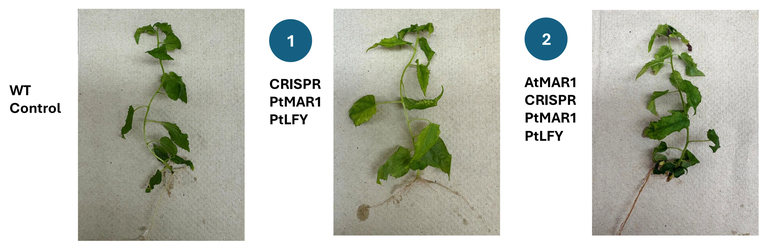



**Sequencing data for two successful knockouts showing variability between events**
Event A has a large deletion flanked by two smaller deletions, and Event B has single base pair deletions in two different sites.

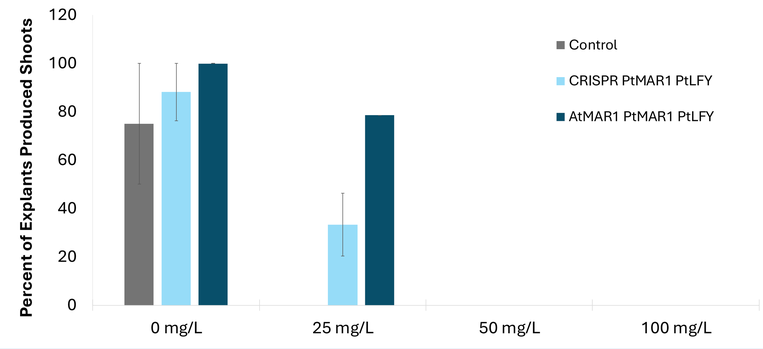
## MAR1 knockouts were less sensitive to kanamycin



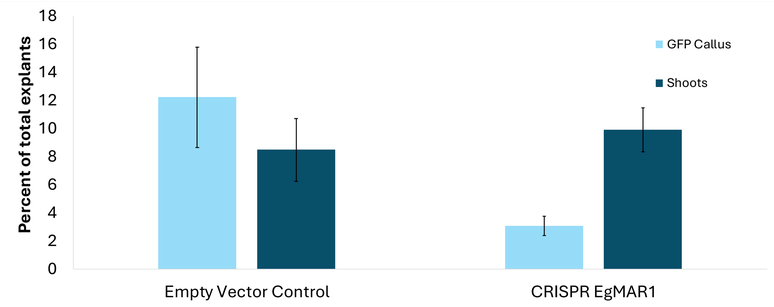

## Transformed explants were phenotypically similar to WT controls in tissue culture




## MAR1 knockouts, but not controls, were resistant to 25 mg/L kanamycin




## Eucalyptus transformation gave rise to transgenic callus but only WT shoots




## Next steps for research

- Test kanamycin sensitivity of transgenic lines and empty vector controls with lower concentrations, to determine the ideal level for co-editing selection in poplar
- Repeat poplar transformation on a larger scale using kanamycin selection. This may lead to isolation of transgene-free edited events
- Modify conditions to enable regeneration of transgenic eucalypt shoots
- Test MAR1 co-editing in other poplar and eucalypt genotypes

## Acknowledgements

We thank GREAT Trees Coop members for support

*Arauco, Eldorado, Suzano, Klabin, and SAPPI*

https://biotechlab.forestry.oregonstate.edu/great-trees-cooperative

## References

Hoengenaert, L., Anders, C., Van Doorsselaere, J., Vanholme, R. and Boerjan, W. (2025), Transgene-free genome editing in poplar. New Phytol, 247: 224-232. https://doi.org/10.1111/nph.20415

Conte, Sarah, et al. "Multiple antibiotic resistance in Arabidopsis is conferred by mutations in a chloroplast-localized transport protein." *Plant physiology* 151.2 (2009): 559-573. https://doi.org/10.1104/pp.109.143487