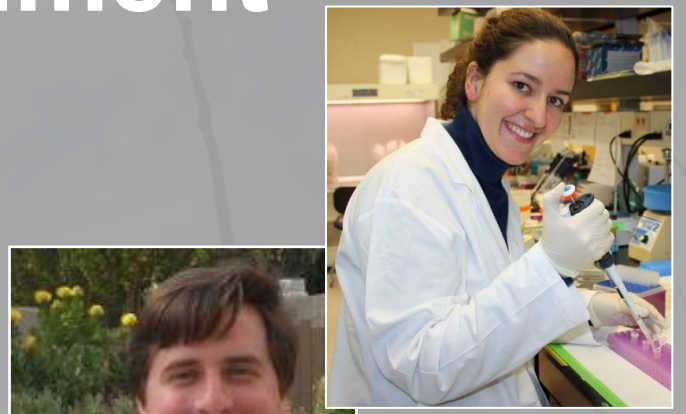
Off-target mutations in CRISPR/Cas9-expressing transgenic trees engineered for containment

Greg Goralogia and Steven H. Strauss

Department of Forest Ecosystems and Society

Oregon State University



Oregon State University

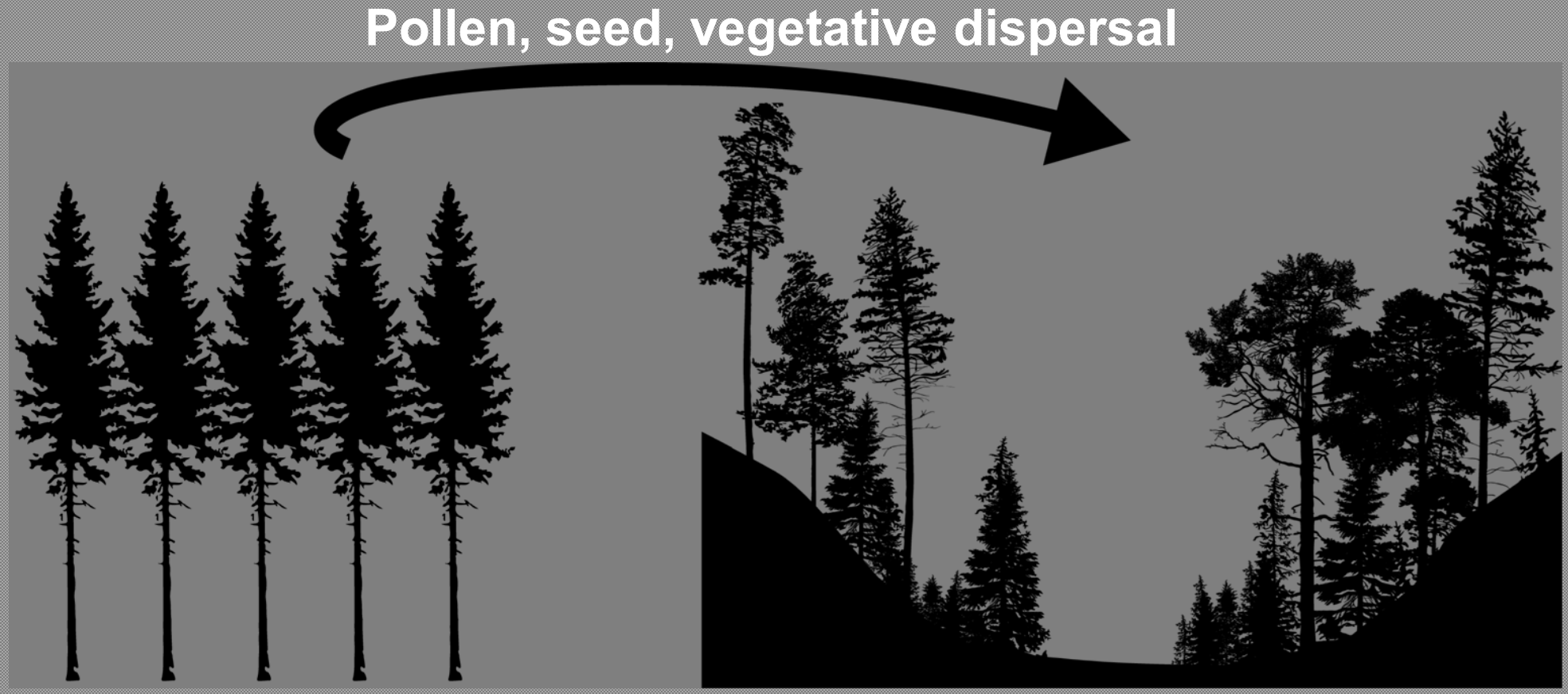
Agenda

- Background, rationale
- Methods
- Results
- Implications

Agenda

- Background, rationale
- Methods
- Results
- Implications

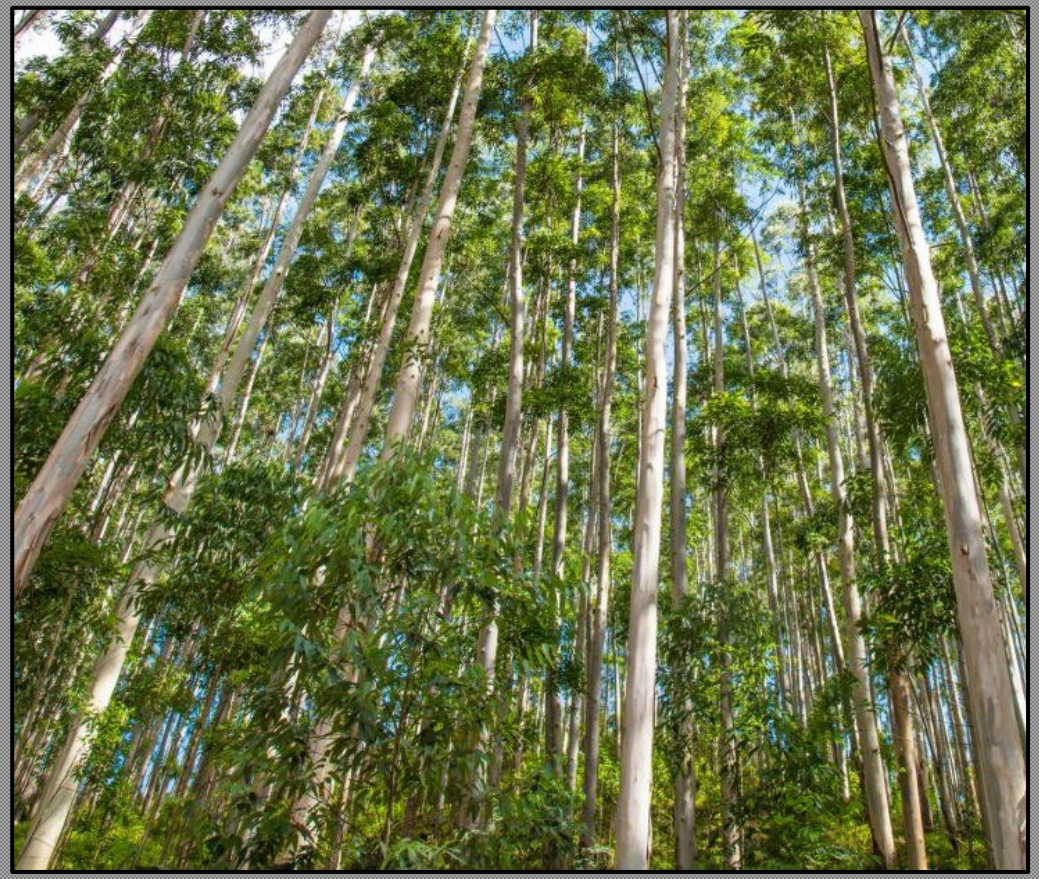
Transgene containment is an important trait in forest biotech at the intersection of science, regulation, and society



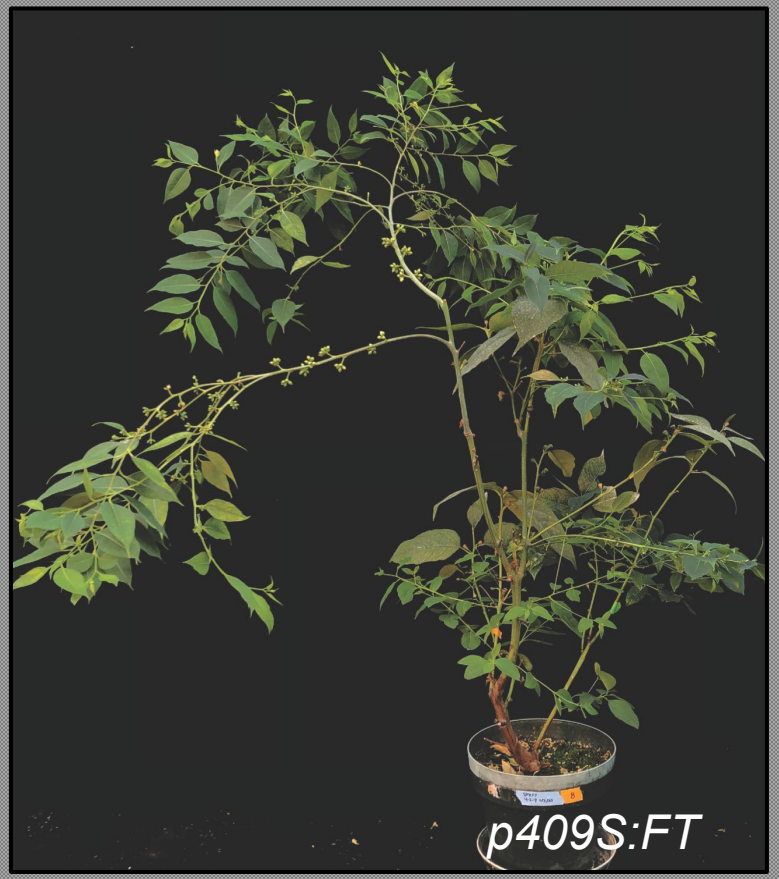
Poplars are a great system for study of gene editing and biocontainment in forest trees

- Easy to transform
- High quality genomes
- Fast growth rate
- Diecious, wind pollinated flowering (but ~3-8 yr. onset)
- In western Oregon, model white poplars sexually incompatible with nearby native poplars (flowering permitted by USDA)

We also work in eucalypt hybrids: Valuable species to global plantation forestry



Eucalyptus grandis x urophylla plantation



Early flowering transgenics to study containment traits

CRISPR/Cas9 is an effective tool to induce reproductive sterility in forest tree species

frontiers in Plant Science

Variation in Mutation Spectra Among CRISPR/Cas9 Mutagenized Poplars

*Estefania Elorriaga[1], Amy L. Klocko[2], Cathleen Ma[1] and Steven H. Strauss[1]**

[1] Department of Forest Ecosystems and Society, Oregon State University, Corvallis, OR, United States, [2] Department of Biology, University of Colorado Colorado Springs, Colorado Springs, CO, United States

Plant Biotechnology Journal Open Access

Plant Biotechnology Journal (2021) **19**, pp. 1743–1755 doi: 10.1111/pbi.13588

Genetic containment in vegetatively propagated forest trees: CRISPR disruption of *LEAFY* function in *Eucalyptus* gives sterile indeterminate inflorescences and normal juvenile development

Estefania Elorriaga[1,a], Amy L. Klocko[2], Cathleen Ma[1], Marc du Plessis[3], Xinmin An[4], Alexander A. Myburg[5] and Steven H. Strauss[1,*]

Edited genes in *Eucalyptus* induce bisexual or male sterility

Removing CRISPR/Cas9 genes in clonally propagated plants is a major challenge

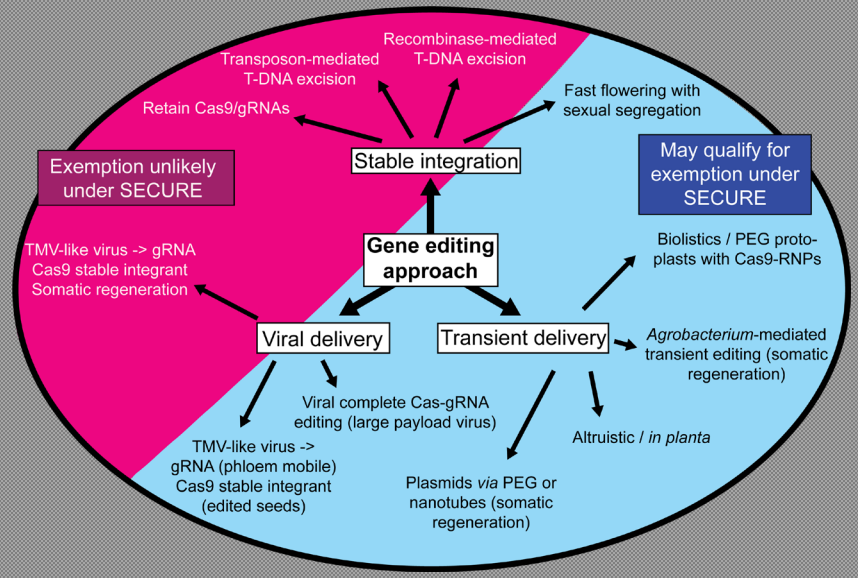
- Hard to segregate (clones, late flowering)
- Cannot fully or efficiently remove transgenic DNA (recombinase)
- CRISPR/Cas9 innocuous? Leave in genome ?
- Are off-target rates acceptable over years?

In Vitro Cellular & Developmental Biology - Plant
https://doi.org/10.1007/s11627-021-10197-x

SPECIAL ISSUE ON GENOME EDITING

Gene editing in tree and clonal crops: progress and challenges

Greg S. Goralogia[1] · Thomas P. Redick[2] · Steven H. Strauss[1]



Ways to get “clean” gene edits in clonally propagated plants

Agenda

- Background, rationale
- Methods
- Results
- Implications

Timeline of study: Plant growth and sampling

3-5 years growth between transformation and off-target study

2019-2020 sampling

Poplars

2014 2015 2017

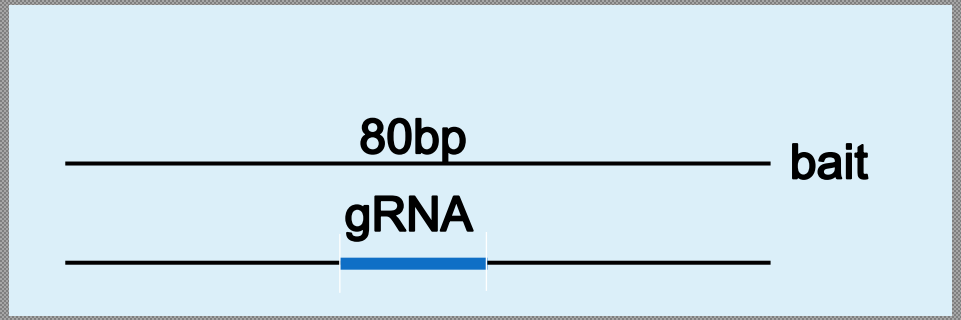
Cloning and construct development | Transformation & event genotyping | Propagation and multiplication | Plants in the field or greenhouse

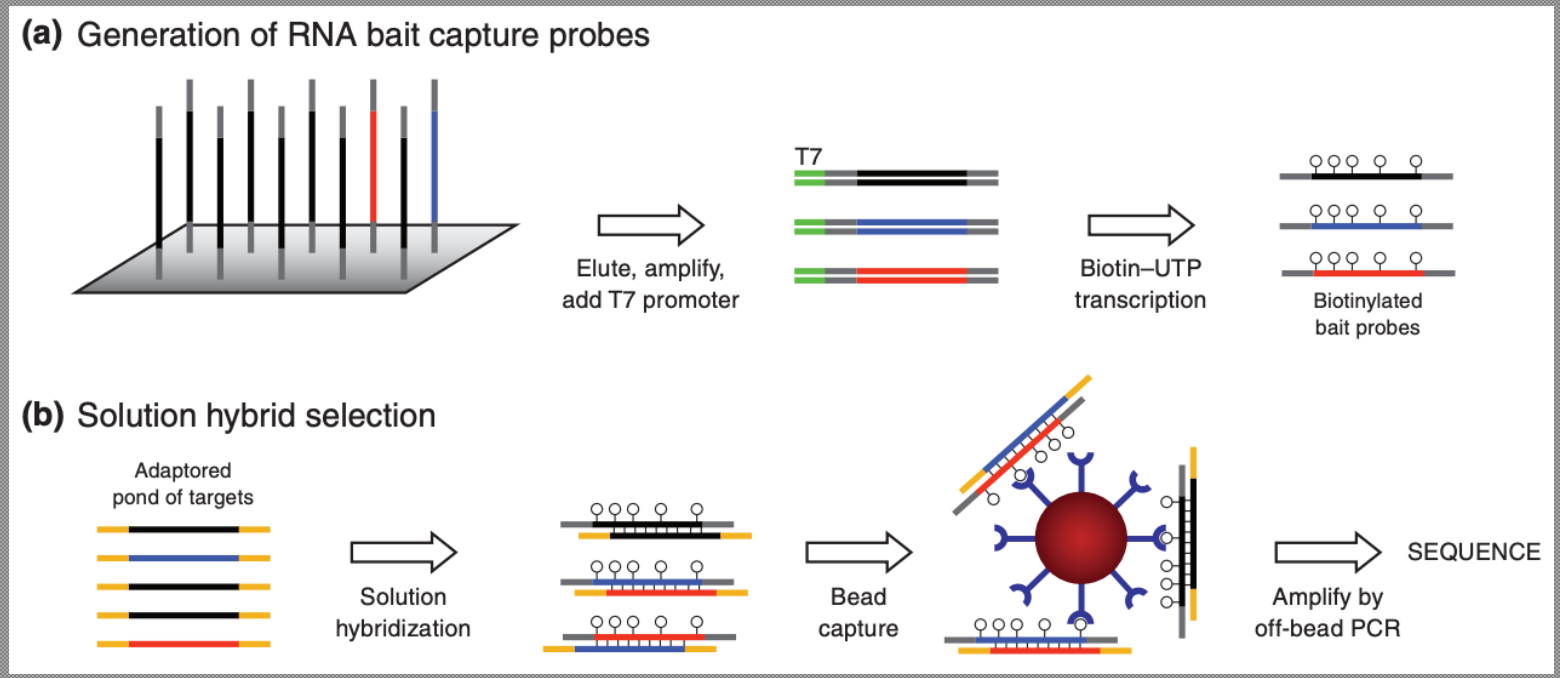
Present day

Eucalypts

2015 2016 2018 2019-2020 sampling

We used a bait-capture approach to survey off-target sites





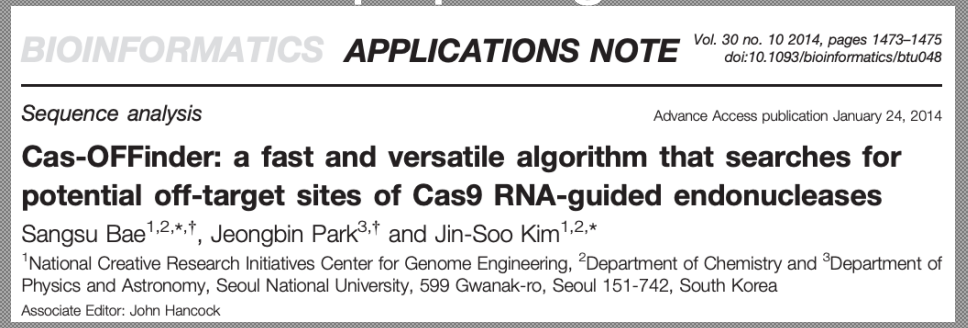
Fisher et al. 2011 *Genome Biol.*

We opted against whole genome sequencing to survey many events at more likely off-target sites

- Limited budget
- Needed high coverage to be confident about mutations at predicted CRISPR/Cas9 off-target sites
- Wanted to sequence as many events as possible comparable to a commercial biotech program, including replication of clonal propagules (ramets)

20,000 probe sites were chosen by degree of mismatch to the target gRNAs (up to 5/20)

- Used Cas-OFFinder software
- ~13,500 sites were designed against the *Populus tremula/alba* genome sand ~6,500 sites for the *Eucalyptus grandis* genome
- 2 recent duplicate *AGAMOUS* genes in poplar, 1 gene for *LEAFY* in poplar and eucalypt
- Mean of 60 to >300 reads per target site
- 1.09 Mbp DNA surveyed by bait capture = 0.3% of the poplar genome



BIOINFORMATICS **APPLICATIONS NOTE** *Vol. 30 no. 10 2014, pages 1473–1475*
doi:10.1093/bioinformatics/btu048

Sequence analysis Advance Access publication January 24, 2014

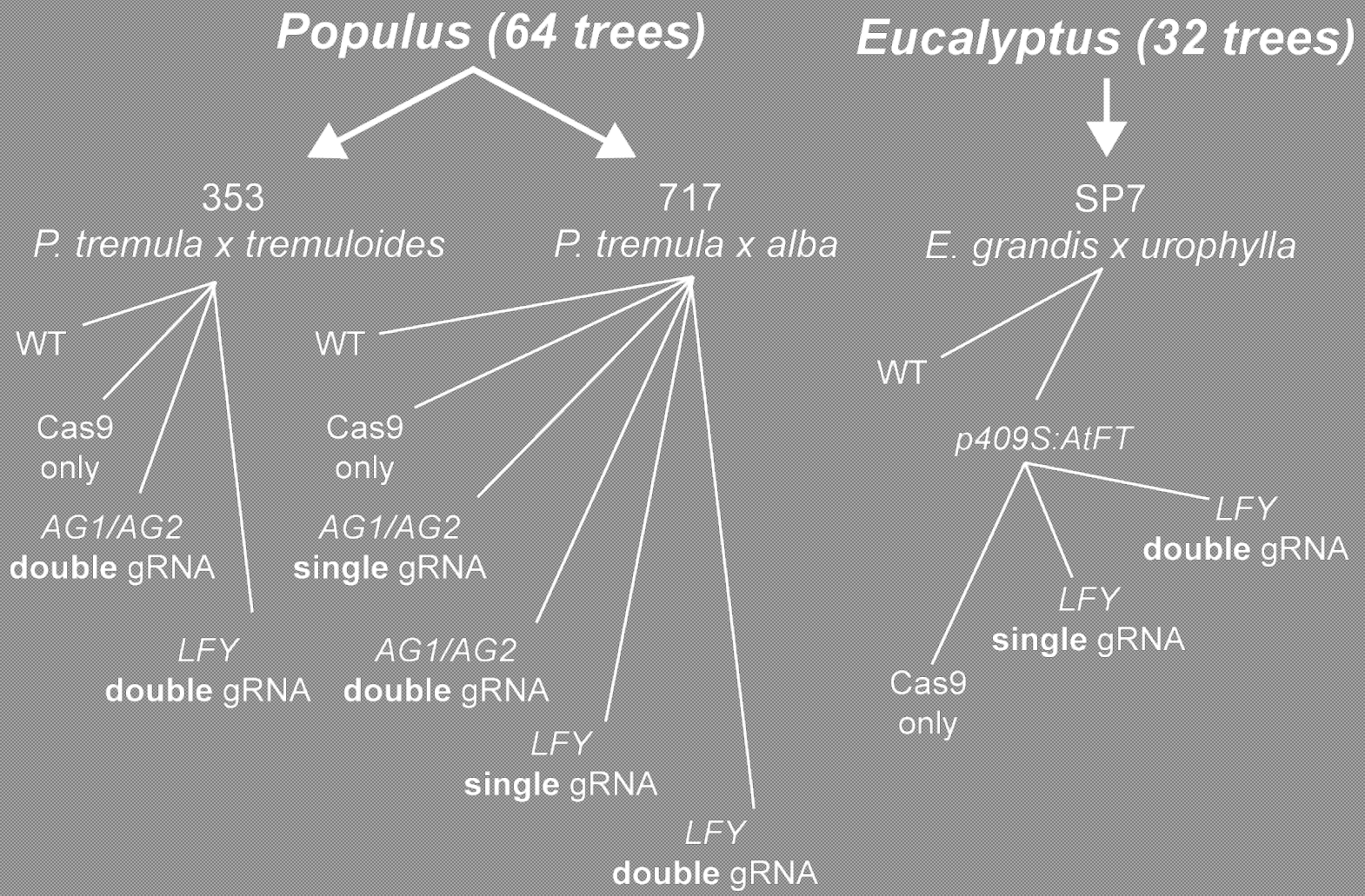
Cas-OFFinder: a fast and versatile algorithm that searches for potential off-target sites of Cas9 RNA-guided endonucleases

Sangsu Bae[1,2,*,†], Jeongbin Park[3,†] and Jin-Soo Kim[1,2,*]

[1]National Creative Research Initiatives Center for Genome Engineering, [2]Department of Chemistry and [3]Department of Physics and Astronomy, Seoul National University, 599 Gwanak-ro, Seoul 151-742, South Korea

Associate Editor: John Hancock

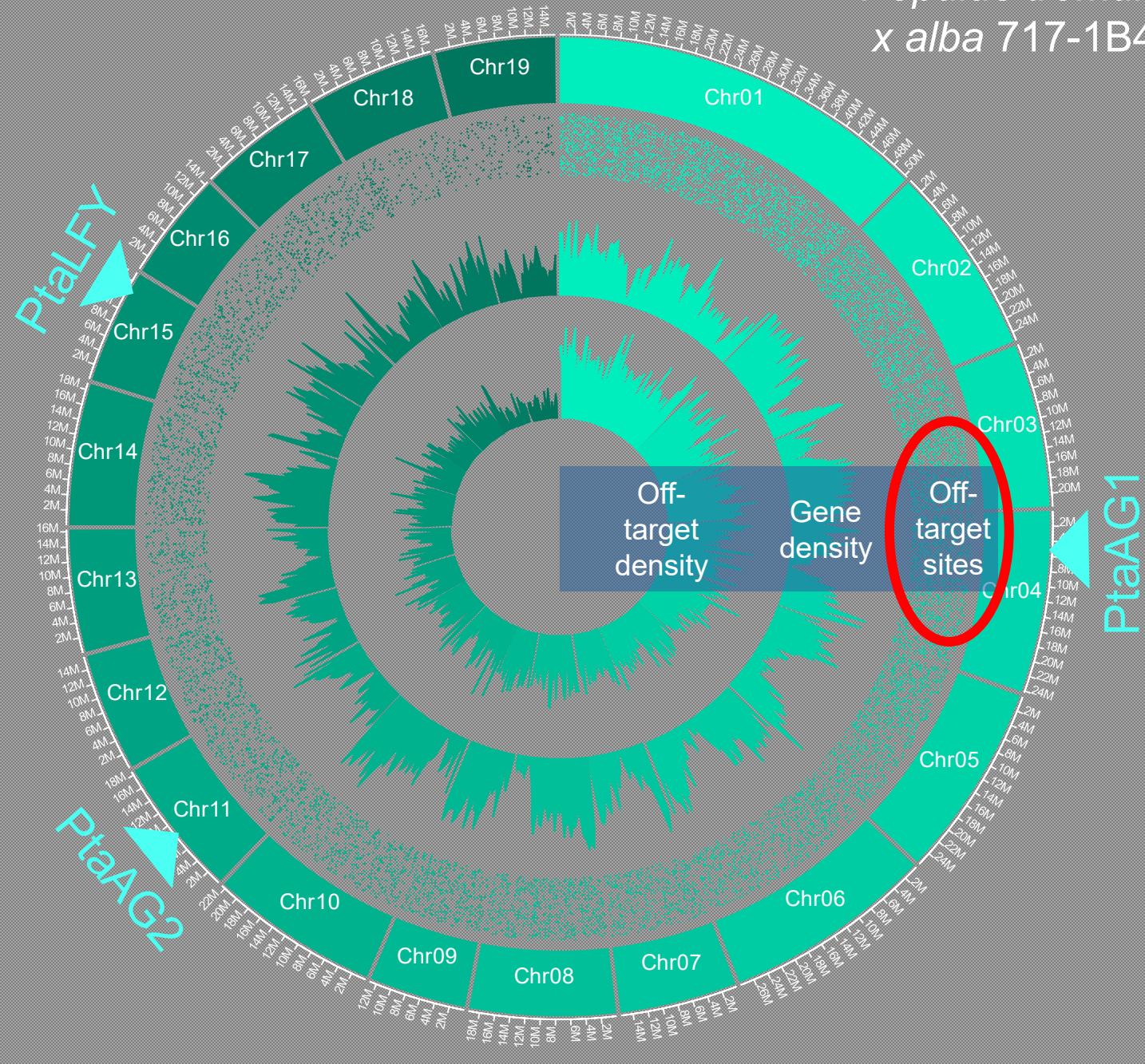
CRISPR/Cas9 constructs targeted *LFY* and *AG* genes with single and double gRNAs, total of 6 unique gRNAs



Population included: biallelic edited events, heterozygous edited events, and transgenic but not edited events

Populus tremula
x alba 717-1B4

Target sites well distributed over the genomes



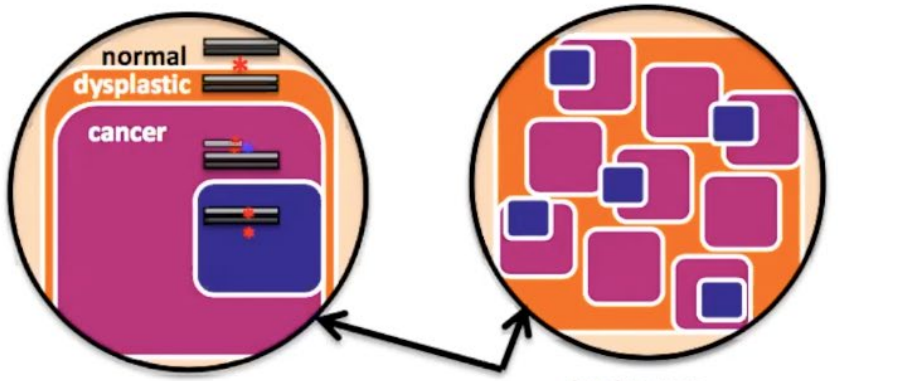
Used Mutect2 program for off-target mutation detection

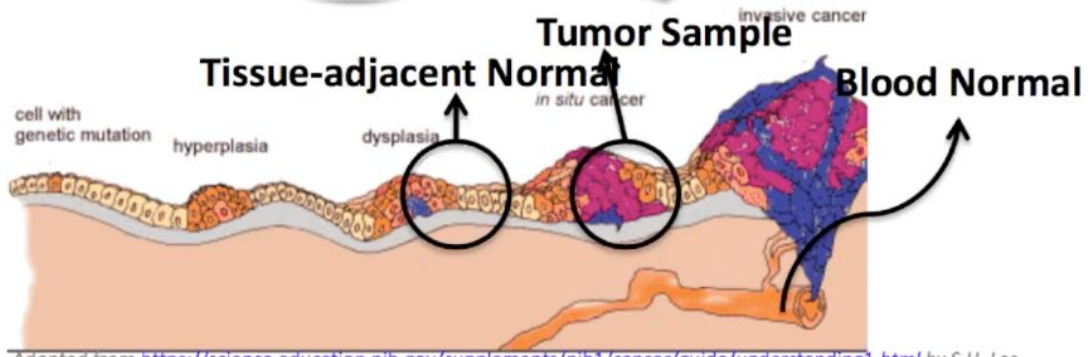
gatk

Tumor and normal contamination and heterogeneity

Tumor **purity** = $\frac{\text{(tumor cells)}}{\text{(normal + tumor cells)}}$

Tumor **heterogeneity** is based on polygenomic populations, segregated or intermixed, due to ongoing subclonal evolution.



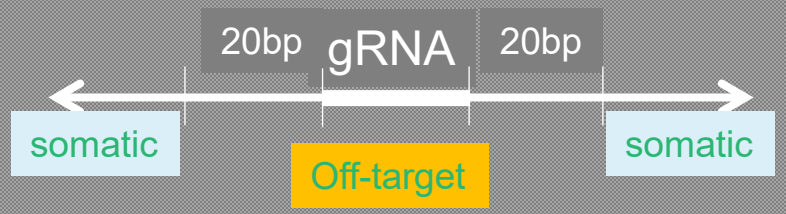


BROAD INSTITUTE

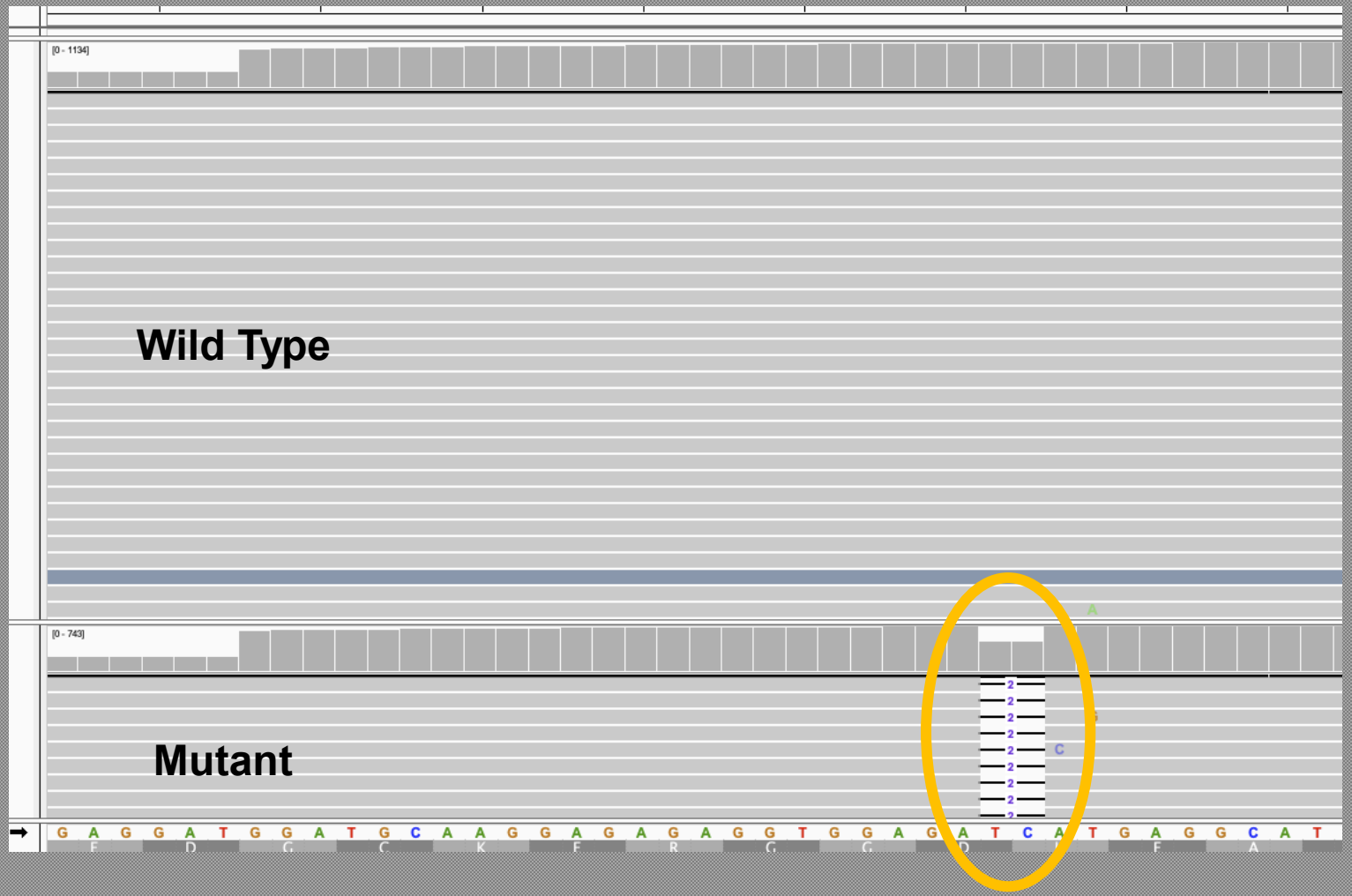
Adopted from https://science.education.nih.gov/supplements/nih1/cancer/guide/understanding1.html by S.H. Lee

Mutant interrogation

- Mutec2 program reports potential mutations at different threshold parameters, then manually inspected
- Need at least 5 reads support
- Must not be a natural polymorphism in our hybrids
- Within target 20bp gRNA-like site = **Off-target mutation**
- At least 20 bp away from ends of the gRNA target = **Somatic mutation**



Manual inspection of Mutect2 reported mutations

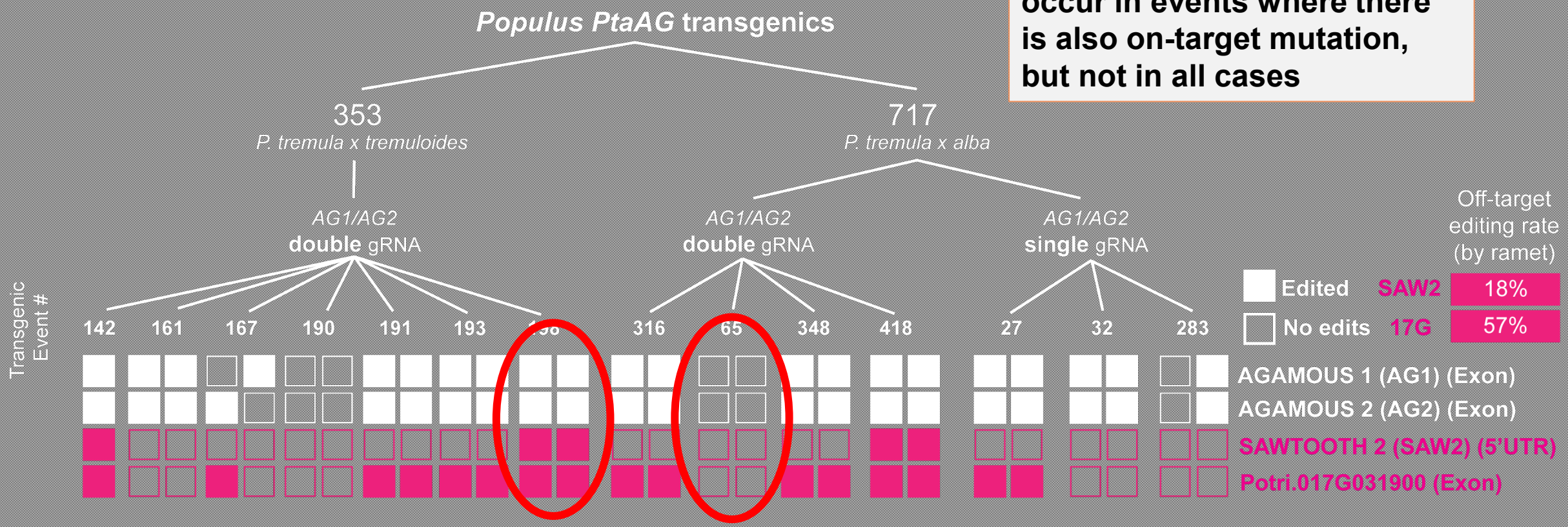


Agenda

- Background, rationale
- Methods
- Results
- Implications

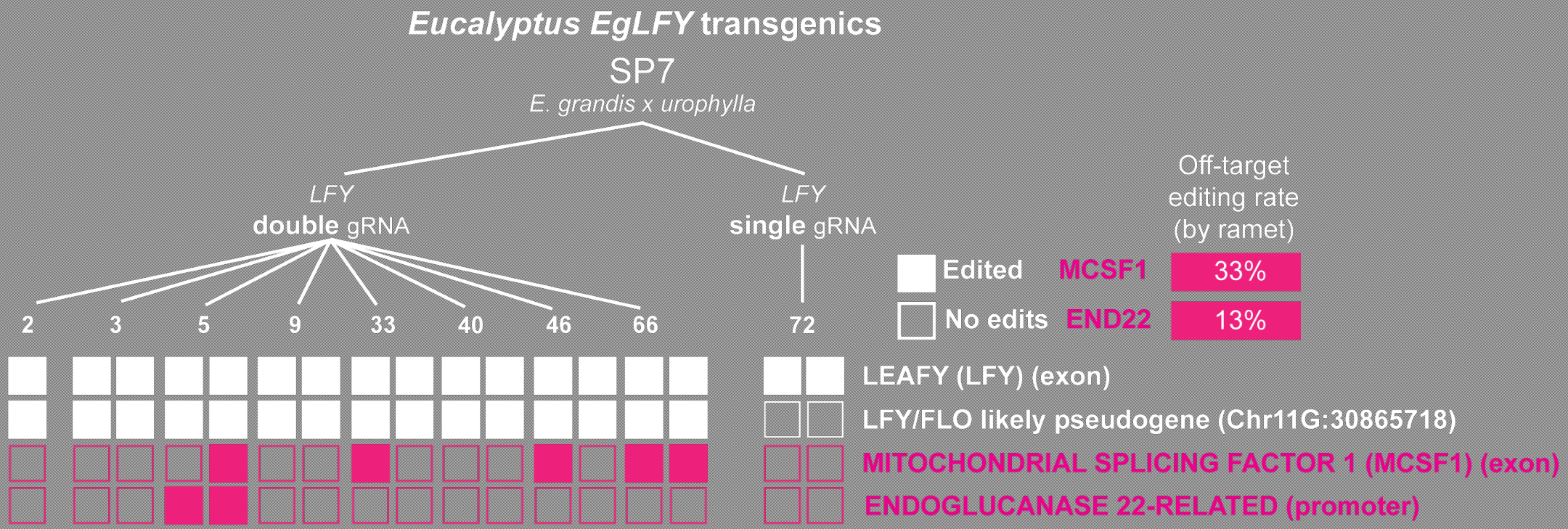
We found two off-target sites mutated in poplars with the *PtaAG* targeting construct, but not with *PtaLFY*

Off-target mutations only occur in events where there is also on-target mutation, but not in all cases



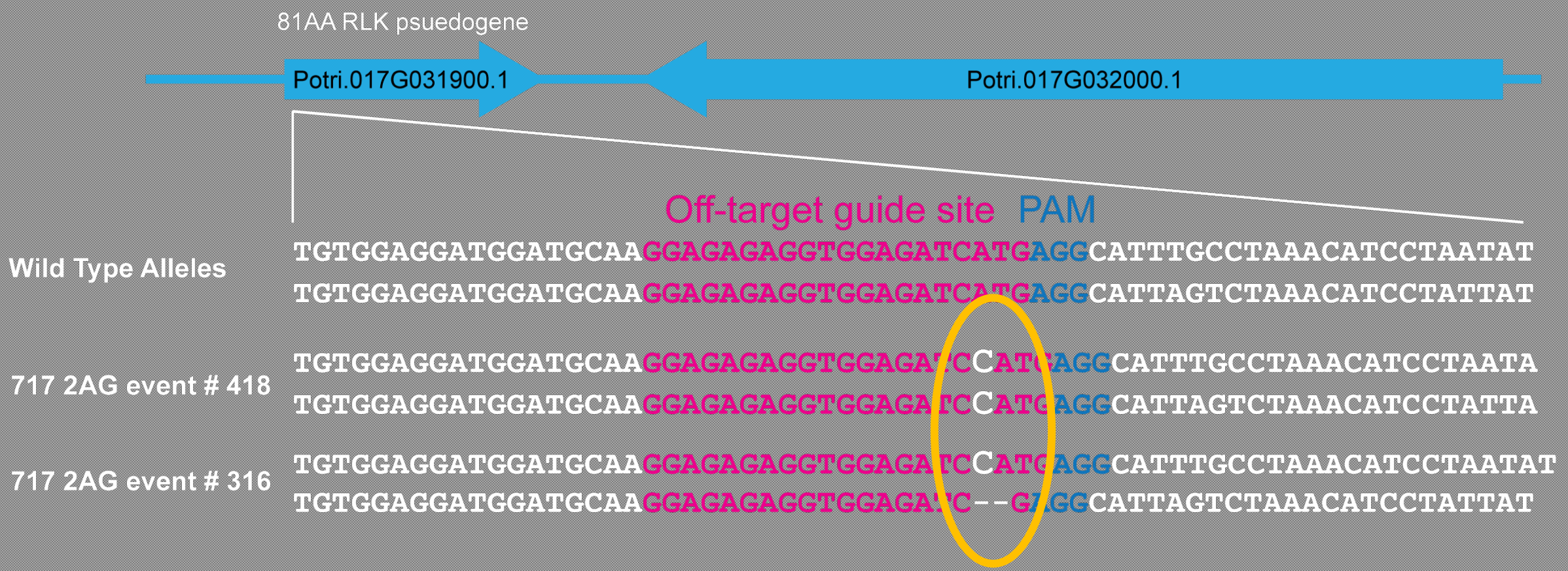
Filled cells = greater than 20% allele frequency

Similarly, we found two off-target mutated sites in eucalypts (targeting *EgLFY*)

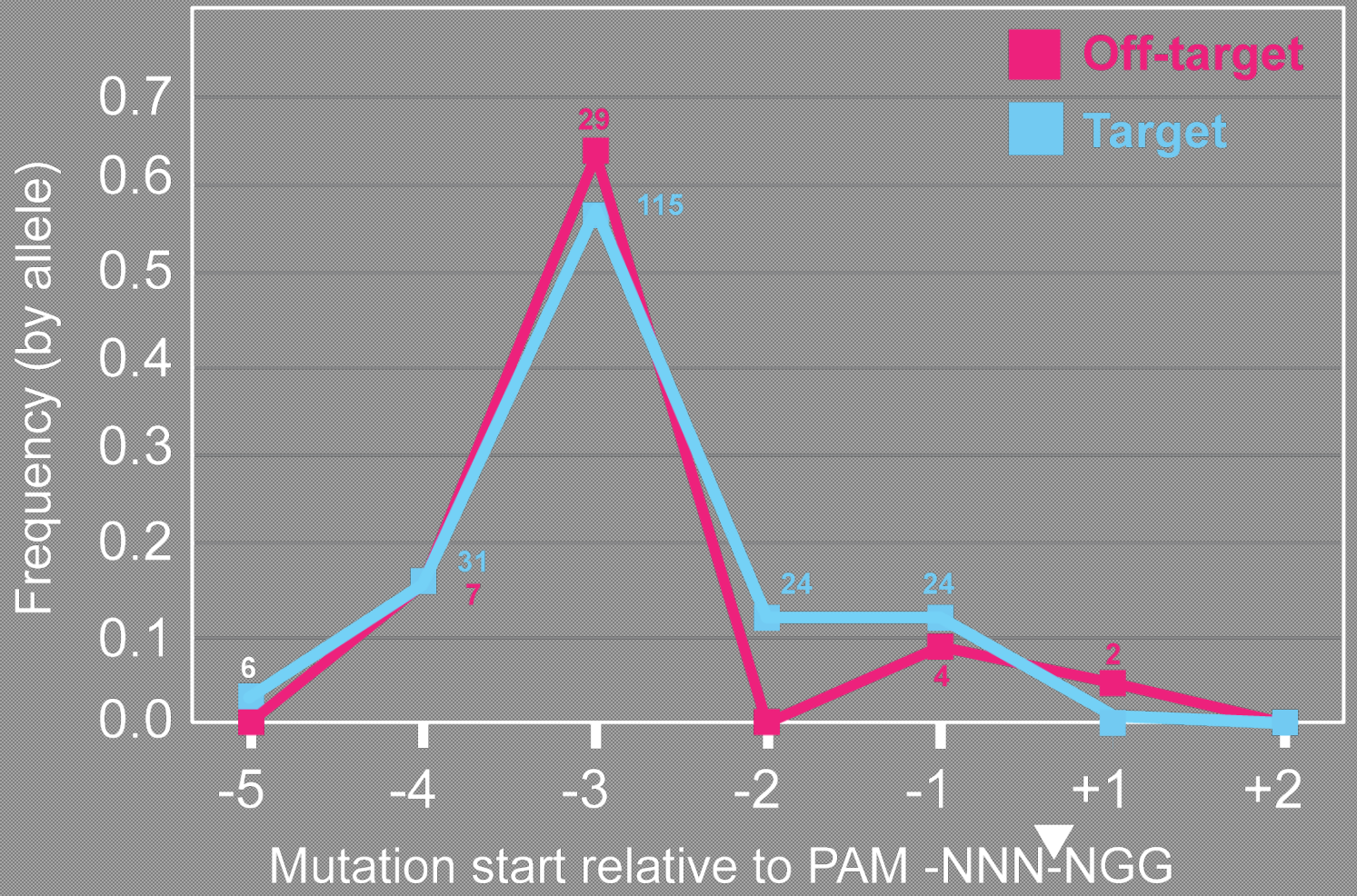


Filled cells = greater than 20% allele frequency

Mutations at off-target loci were small indels proximal to PAM site, as expected for Cas9

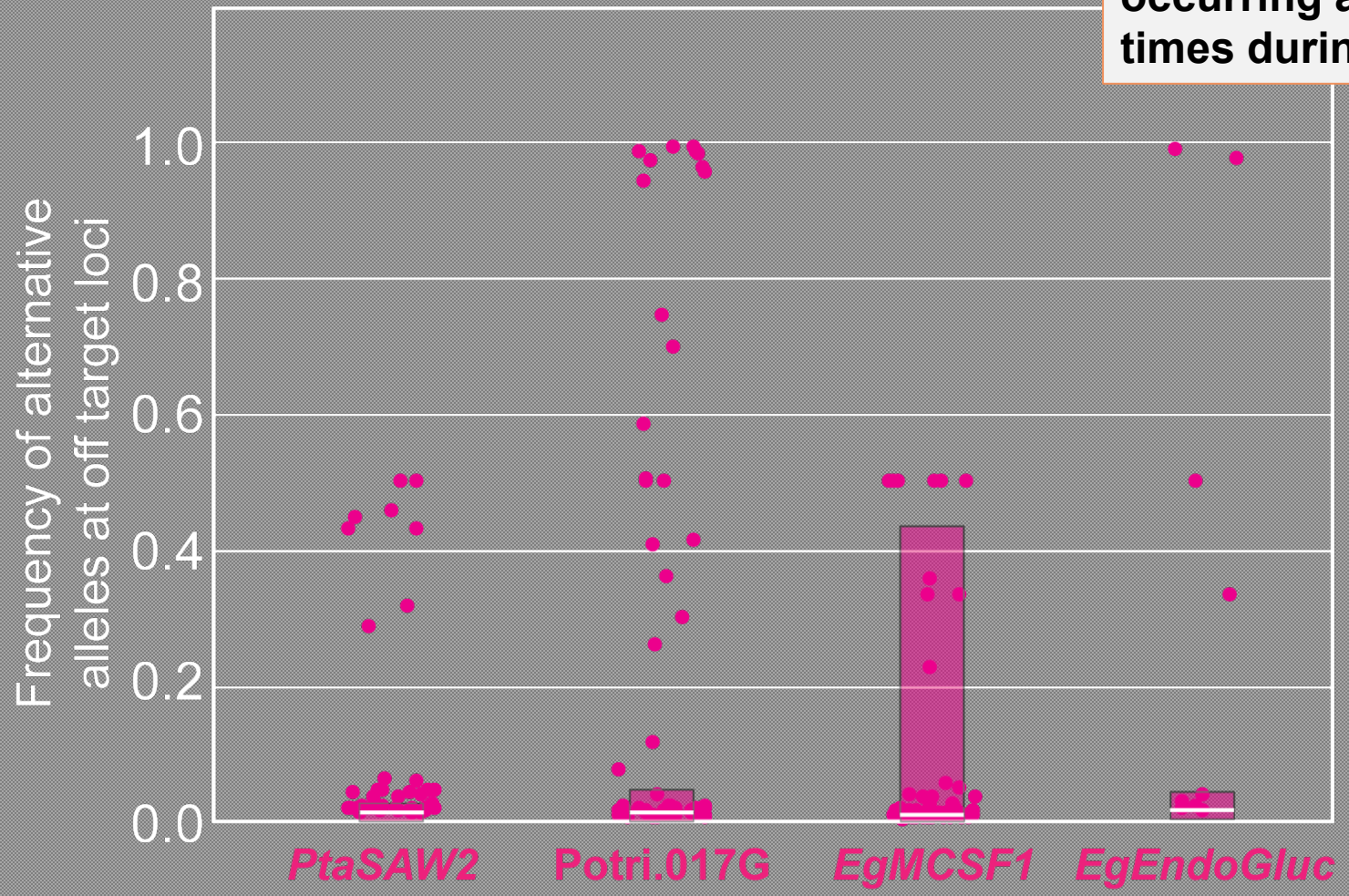


The distance from the PAM to the induced mutation was the same for on- and off-target sites



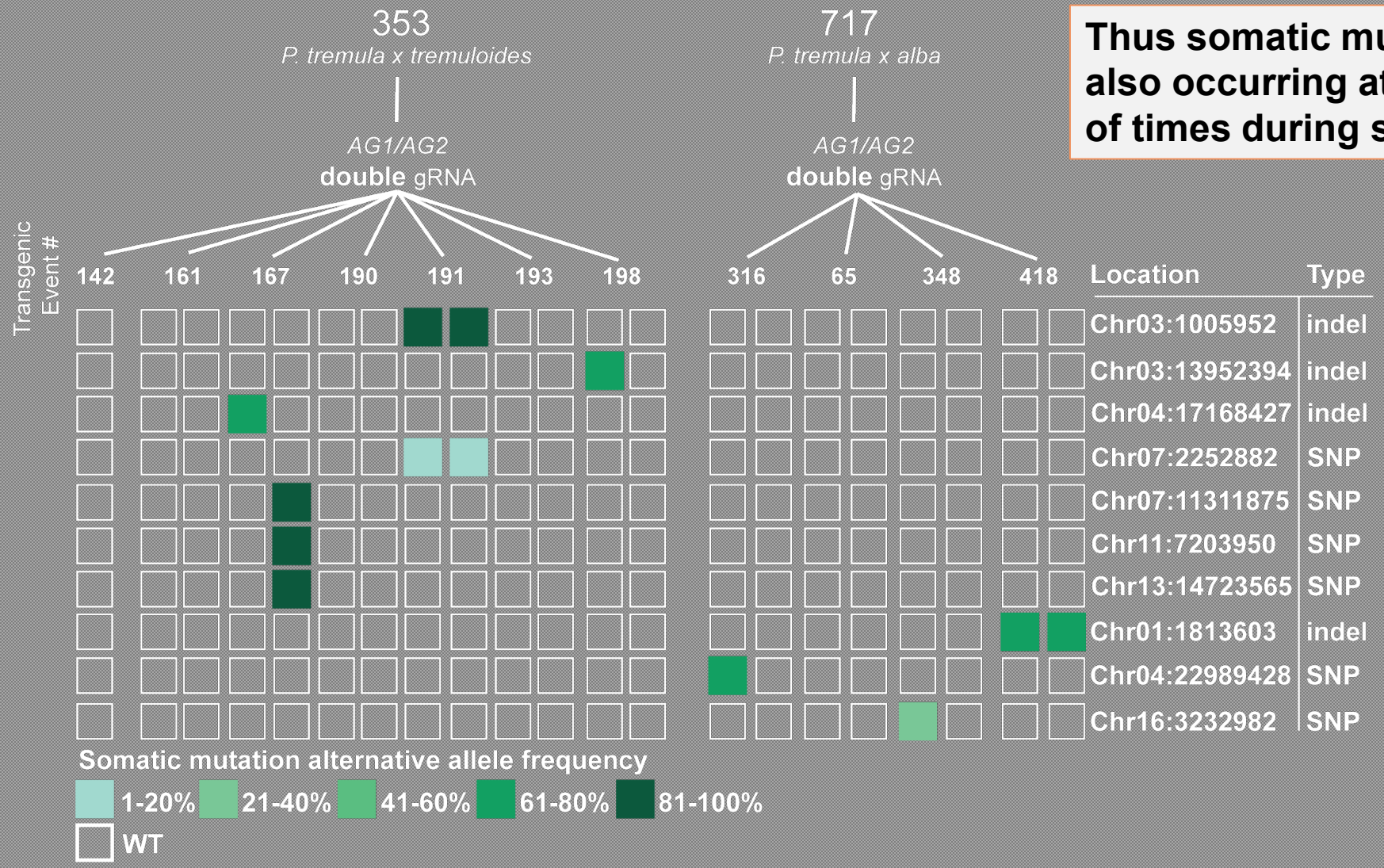
Frequencies of edited alleles at each site varied widely, some reached fixation

Thus mutations were occurring at a wide variety of times during somatic growth



Off-target sites often quite divergent from sequence of sgRNA

Somatic mutations found within many events and even single ramets, frequency also highly variable



Thus somatic mutations were also occurring at a wide variety of times during somatic growth

Takeaways: We observed some mutagenic gRNAs, but off-target mutation rates *extremely low*

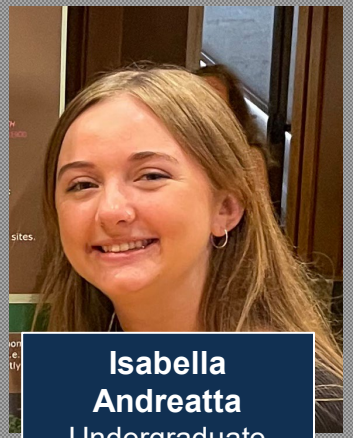
- High rates of off-target mutation at a few loci in many independent events suggest that some loci have high binding affinity for the Cas9/target guides
- Off-target rates we found were predicted to occur at $2x10^{-9}$ bases in poplar, and less in eucalypts
- Reported rates of sexual mutation range from 7×10^{-9} (Arabidopsis) to 3×10^{-8} (maize) per generation – **so very similar or lower than background rate expected in breeding**

Some caveats and directions

- Few sgRNAs (6) targeting 4 independent genes, only 2 of these had off-target mutations observed
  - *Mutation rates are very heterogeneous among targets and events -- A narrow sample of targets studied*
  - *Screening larger numbers of events and targets, at depth, desirable in future work*
- Reason why some targets are much more prone to mutations than others is unclear – needs biophysical study?
- The edited trees are coming into flower, and will be studied for possible chimerism and for phenotypic effects – both for flowering and vegetative growth
- Means for efficient excision of CRISPR/Cas in development

Thanks to many, over many years

Greg the lead on this work



Isabella Andreatta
Undergraduate Researcher

Kelly Vining
Professor, OSU Horticulture, coPI

Steve Strauss
PI, Professor

Amy Kloko
Assistant Professor UCCS, former Postdoc

Estefania Elorriaga
former PhD student, now Postdoc

Cathleen Ma
Transformation & Greenhouse Experiments

Amanda Goddard
Program & Field Manager

- USDA Biotechnology Risk Assessment Grant (BRAG), # 2017-03820
- GREAT TREES industrial Cooperative