Low frequency and deleterious impacts from zinc finger nuclease mutagenesis in *Populus*

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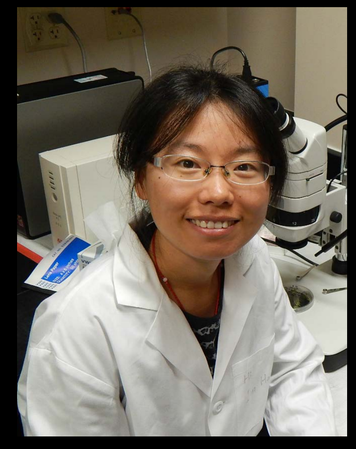


Abstract

Gene flow from recombinant-DNA modified (GMO) trees is a major barrier to their public acceptance and regulatory approval. Because many intensively grown trees are vegetatively propagated, complete sexual sterility could be a powerful means to mitigate or prevent gene flow. We tested four pairs of zinc-finger nucleases (ZFNs) as mutagenic agents against the *LEAFY* and *AGAMOUS* orthologs in poplar that are expected to be required for sexual fertility. To reduce the potential for pleiotropic effects from mutagenesis, each of the pairs was cloned behind a heat-shock promoter that drove ZFN expression. Using *Agrobacterium tumefaciens*, we transformed more than 21,000 explants. The rate of transformation was substantially reduced by the ZFN constructs; only 391 transgenic shoots were produced (1.8 %). After heat shock and subsequent development of the transgenic plants, only two events were found to contain mutations; both were 7-bp deletions in one allele of the *PtAG2* locus. No mutations were observed in the *PtAG1* or *PtLFY* loci. Our results indicate a mutation rate of zero to 0.3% per explant per allele, among the lowest reported for ZFN mutagenesis in plants. The combined effects of low recovery of transgenic plants, a modest mutation frequency, and much higher reported rates of directed mutation for other gene editing methods, suggests that technical issues regarding the use of ZFNs in poplar still need to be resolved for full reduction of the technology to practice.

Experimental strategy

(1) Created and validated four ZFN pairs to target *LEAFY* or *AGAMOUS* genes in poplar (*i.e.*, *PtLFY*, *PtAG1*, and *PtAG*2)
(2) Constructed a heat-inducible vector system and tested two heat shock treatment methods to control ZFN expression
(3) Regenerated transgenic poplars with *Agrobacterium*-mediated transformation
(4) Screened for mutations in ZFN-transgenic poplars using high resolution melting (HRM) to determine their frequency
(5) Sequenced HRM-predicted mutations to determine their nature (*e.g.*, SNPs, deletions, and insertions)

Four ZFN pairs were created and validated *in vivo*

Collaborating with DOW AgroSciences, we created and selected two ZFN pairs (ZFN800 and ZFN801) that target the MADS-box region in both *PtAG* paralogs and two ZFN pairs (ZFN802 and ZFN803) that target *PtLFY*. All of the four ZFN pairs were validated *in vivo* (mice and yeast) by DOW AgroSciences.

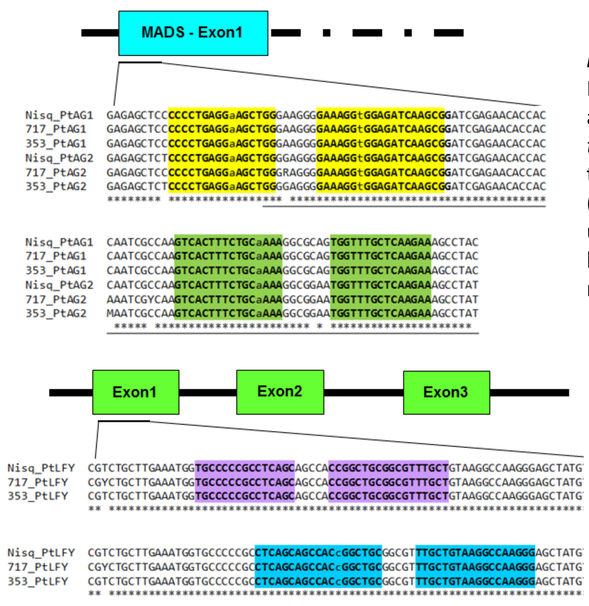


***Fig.* 1. ZFN targets in *PtAGs*.** The reference genomes Nisq_AG1-MADS (Potri.004G064300, v3 of *P. trichocarpa*) and Nisq_AG2-MADS (Potri.011G075800, v3 of *P. trichocarpa*) are given above the sequences from the target transformation hosts 717-1B4 (hereafter 717) and 353-53 (here after 353). The region that includes the MADS-box is underlined. The target sites of ZFN800 and ZFN801 are in bold and highlighted in yellow and green respectively; the nucleotides in lowercase are not targeted by the ZFNs.

***Fig.* 2. ZFN targets in *PtLFY*.** The reference genome Nisq LFY (Potri.015G106900, v3 of P. trichocarpa) is given above the sequences from the target transformation hosts 717 and 353. The target sites of ZFN802 and ZFN803 are highlighted in violet and blue respectively; note the nucleotides in lowercase are not targeted by ZFNs.

Heat-inducible vector system

Within each ZFN pair, the two ZFNs are joined by a 2A linker that contain nuclear localization sequences (NLSs). Each of the four ZFN pairs was cloned into the same backbone vector with a *HSP* gene (derived from the soybean heat-inducible promoter *HSP6871*) that drove *ZFN* expression. The vector also had a constitutive *GFP* gene as a reporter to help us non-destructively monitor transformation and chimerism. A *HSP:GFP* gene vector was also transformed into poplar to study kinetics of heat induction.

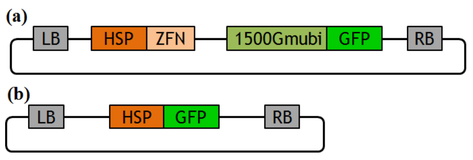
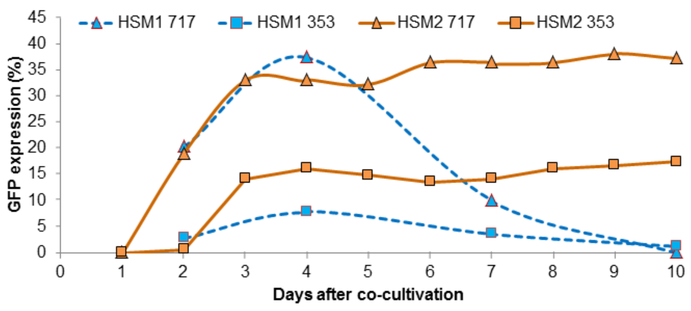


***Fig.* 3. Vectors for poplar transformation.** (a) Schematic diagram of the HSP:ZFN-1500:GFP vector, which was used for producing ZFN-transgenic poplar. (b) Schematic diagram of the HSP:GFP vector, which facilitated study of heat induction kinetics in transgenic poplar.

Two heat shock methods for inducing transgene expression

We compared two heat shock methods for their efficiency with HSP:GFP-transformed explants. In both tested clones, one method provided a ZFN "pulse", while the other provided a prolonged period of nuclease delivery. Both of these methods were used in producing ZFN-transgenic poplars.



- **Pulse Heat Shock Method (HSM 1)**: Incubate explants at 42°C for 16 hrs, then shift them to 22°C to recover for 32 hrs, and incubate them a second time at 42°C for 16 hrs. – *less prone to chimerism?*
- **Prolonged Heat Shock Method (HSM 2)**: Incubate explants at 42°C for 3 hrs every day for 10 days. – *more mutants?*

***Fig.* 4. The percentages of *GFP*-expressing explants upon heat shock.** In both 717 and 353, HSM 1 induced a pulse of GFP expression, while HSM2 induced much higher GFP expression. For each heat shock method, data was developed from studies with sample size n>180.

GFP expression to monitor ZFN T-DNA delivery

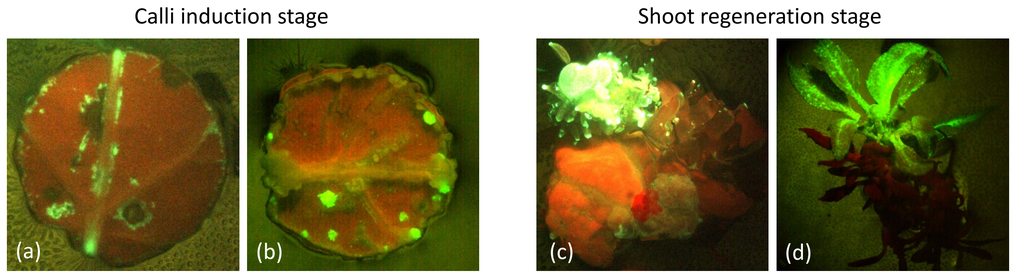


***Fig.* 5. *GFP* expression observed under dissecting microscope.** The constitutive *GFP* gene in our ZFN constructs helped to non-destructively monitor transformation and chimerism. The GFP signal was recorded during organogenesis : (a) after 2 days on calli induction medium, (b) after 3 weeks on calli induction medium, (c) after 3 weeks on shoot regeneration medium, and (d) after 8 weeks on shoot regeneration medium.

Regeneration and confirmation of ZFN-transgenic events

The four ZFN constructs were transformed into *A. tumefaciens* strain AGL1 and then transformed into poplar clones 717 and 353. All of the culture media contain kanamycin for transgenic tissue selection, and timentin for selection against *A. tumefaciens*. After two months in root induction medium, *in vitro* propagated candidates of transgenic poplars were harvested for GFP signal checking, DNA extraction and PCR confirmation.

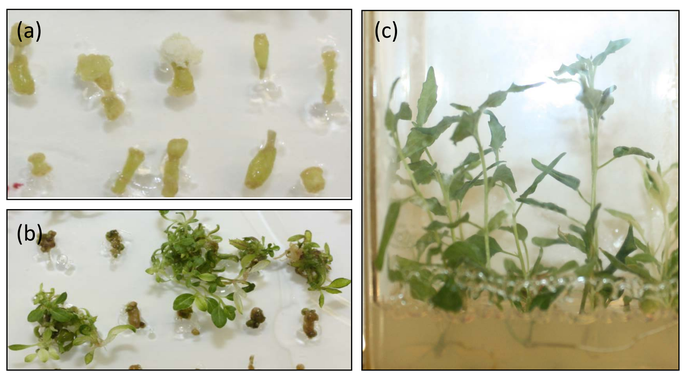


***Fig.* 6. *in vitro* regeneration of ZFN-transgenic poplar.** ZFN800-treated plants on selective (a) calli induction medium, (b) shoot induction medium and (c) rooting medium. Shoot tips from rooted events were collected and checked for GFP signal. PCR amplification of transgene was also used for ambiguous shoots.

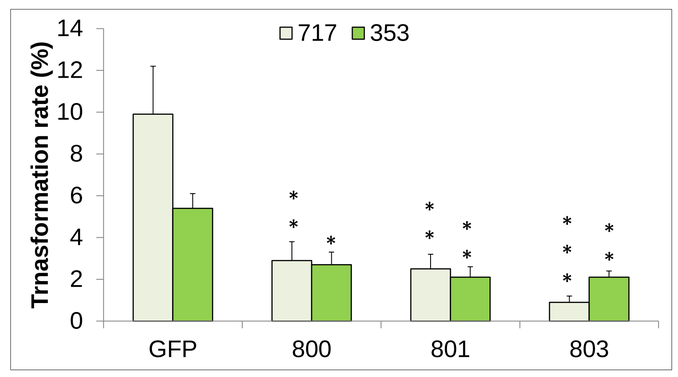
Low shoot regeneration and transformation rates with ZFNs

For each ZFN construct and each poplar clone, we co-cultivated over 2,000 explants. In total, we were able to get the 2,206 regenerated shoots. However, only 391 ZFN-transgenic events were confirmed by GFP expression and/or PCR, and all of them were produced from the "pulse" heat shock method. The reduced transformation rate might be a result of ZFN expression. Additionally, we were unable to get any regenerated ZFN802-transgenic events. PCR analysis of the *Agrobacterium* strain that was used for plant transformation showed an absence of the ZFN transgene, despite multiple efforts to transform it. We suspect this ZFN pair is toxic to the bacteria thus it could not effectively transform plants.

Construct	No. of transformed explants		No. of explants that regenerated shoots		No. of confirmed ZFN-transformants	
	717	353	717	353	717	353
ZFN800(PtAGs)	3,126	3,315	772	206	119	80
ZFN801(PtAGs)	2,140	2,085	229	234	39	30
ZFN802 (PtLFY)	2,231	2,271	1	8	0	0
ZFN803 (PtLFY)	3,431	3,099	594	162	72	51
Total	**21,698**		**2,206**		**391**	

***Table* 1. Transformation and regeneration of ZFN-transgenic poplars.** Despite the cultivation of 21,698 explants, a much smaller than expected number of regenerated (2,206) and transgenic (391) shoots were produced.

***Fig.* 7. Transformation rates varied widely among three ZFN constructs.** Explants transformed with ZFN constructs showed an overall shoot regeneration rate of 9.6% (ZFN 802 excluded) and an overall transformation rate of 2.2% (ZFN 802 excluded), both of which were lower than those from the HSP:GFP construct (20.4% and 5.8%, respectively), suggesting deleterious effects of ZFNs on cell viability. The modest transformation rates with HSP:GFP also indicate a negative effect of heat shock treatment on plant growth. Bars denote standard errors over four to six independent transformation tests. Comparisons were to GFP construct . ***, p <0.001; **, p <0.01; *, p <0.05



Detection and characterization of ZFN-induced mutations

High resolution melting (HRM) is a PCR-based method for detecting DNA sequence polymorphisms or mutations. It detects variants based on *Tm* values and the shape of the melt curves. Using this method, we screened all of the 391 ZFN-transgenic events for alterations to *PtAG*1, *PtAG*2 or *PtLFY* using locus specific primers. Two putative mutations were found among the 199 ZFN800-transgenic events. No mutations were found in ZFN801- or ZFN803-transgenic events. TOPO cloning and sequencing analysis revealed that both ZFN800-induced mutants contained deletions in one allele of *PtAG2*, suggesting a mutation rate of 0.3% per explant per allele for this ZFN pair.

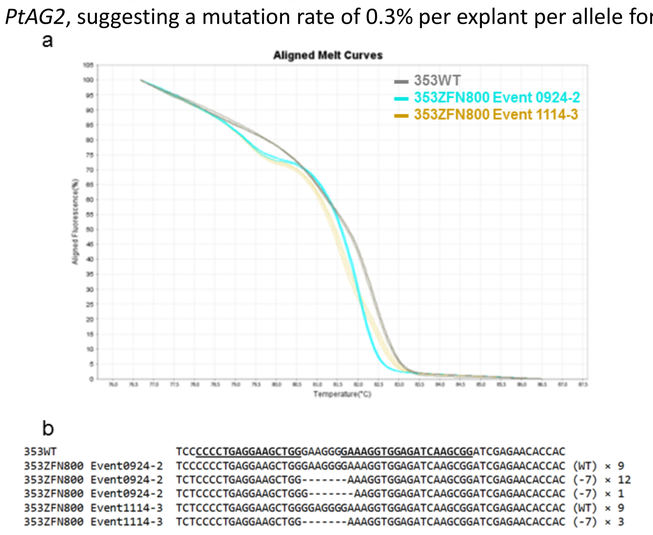


***Fig.* 8. Mutation detection and characterization by HRM and sequencing.** a The putative mutants, Event 0924-2 and Event 1114-3, showed different melt curves from wild type (WT). b Sequencing results revealed deletions in *PtAG2* in the two mutants. Sequence of *PtAG2* from WT 353 is shown on top; left and right target sites of ZFN800 are underlined; numbers within parentheses show how many base pairs were deleted; numbers following parentheses indicate how many times each sequence was observed among independently cloned amplicons.

Conclusions

We studied the frequency of ZFN-induced mutagenesis in *Populus* by testing four heat inducible ZFN constructs that target two floral genes, *PtAGs* or *PtLFY*. Unfortunately, the rates of mutagenesis were low and the deleterious impacts of the ZFNs on transformation rate were high. Heat-induction also reduced overall rate of transformation very significantly. Substantial modifications to system design, and most likely use of alternative SDNs such as CRISPR/Cas9, are needed to achieve useful rates. This conclusion is relevant to research — where many knock-outs or modifications are normally required for replication, and to commercial applications — where many transformation events are typically screened and many background genotypes deployed.

ACKNOWLEDGMENTS

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