Mol Breeding (2016) 36:121
DOI 10.1007/s11032-016-0546-z

Low frequency of zinc-finger nuclease-induced mutagenesis in *Populus*

Haiwei Lu · Amy L. Klocko · Michael Dow · Cathleen Ma · Vindhya Amarasinghe · Steven H. Strauss

Received: 13 July 2016 / Accepted: 12 August 2016 / Published online: 18 August 2016

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 functionally linked to a heat shock promoter to provide inducible ZFN expression. Using *Agrobacterium tumefaciens*, we transformed more than 21,000 total explants compromised of both male and female hybrid poplar. The rate of transformation for the ZFN constructs (2 %) was generally reduced compared to the transgenic control (8 %). We produced 391 ZFN transgenic shoots of which only two developed into plants with mutations in a target gene; 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 suggest that the efficient use of ZFNs in poplar requires further technical improvements.

Keywords Genetic engineering · Forest biotechnology · *AGAMOUS* · *LEAFY* · Gene editing

Electronic supplementary material The online version of this article (doi:10.1007/s11032-016-0546-z) contains supplementary material, which is available to authorized users.

H. Lu · A. L. Klocko · C. Ma · S. H. Strauss (✉)
Department of Forest Ecosystems and Society, Oregon State University, Corvallis, OR 97331-5752, USA
e-mail: Steve.Strauss@oregonstate.edu

Present Address:
M. Dow
PO Box 237, Ashton, SA 5137, Australia

Present Address:
V. Amarasinghe
Department of Botany and Plant Pathology, Oregon State University, Corvallis, OR 97331-5752, USA

Introduction

Transgene dispersal from genetically engineered (GE) crops and trees poses ethical, ecological, and agronomic concerns to various segments of the public (Stewart et al. 2003; DiFazio et al. 2012). In part because of these concerns, GE crops and trees are stringently regulated in the USA (Bennett et al. 2013) and across the world (Dunwell 2014; Li et al. 2014). Such regulations have severely hindered field research and commercialization (Viswanath et al. 2012), including for trees that are important to forest health

or that resemble the products of conventional breeding (Strauss et al. 2009, 2015). Because many intensively grown trees, such as *Eucalyptus* and *Populus*, are vegetatively propagated, one option to mitigate these concerns is bisexual sterility (Brunner et al. 2007). This may also result in more vigorous vegetative growth and yield, and reduced levels of allergens from tree pollen, in some species (Strauss et al. 1995; Hoenicka et al. 2012; Moriguchi et al. 2014). Several types of sexual sterility systems have been developed (reviewed by Strauss et al. 2004; Brunner et al. 2007; Vining et al. 2012) and some have been tested in tree species under field conditions. For example, the barnase-based floral organ ablation strategy has been shown to be efficient and stable in inducing male sterility in field-grown *Pinus*, *Eucalyptus*, and *Populus* trees for multiple years (Zhang et al. 2012; Elorriaga et al. 2014). However, this method can have negative impacts on vegetative growth (*e.g.*, reported by Elorriaga et al. 2014) and does not address the desire for seed as well as pollen containment. Also current strategies largely rely on gene expression or suppression, and thus, their efficiency may vary over time due to gene silencing, especially when considering the long life span of tree species. A method based on mutagenesis of essential floral genes should provide more reliable, if not permanent, bisexual sterility.

Mutagenesis of specific genes in plants has been enabled by site-directed nucleases (SDNs), such as meganucleases (MNs), zinc-finger nucleases (ZFNs), transcription activator-like effector nucleases (TALENs), and the clustered regularly interspaced short palindromic repeats (CRISPR)/Cas9 nuclease system (reviewed by Belhaj et al. 2013; Osakabe and Osakabe 2014; Ain et al. 2015). These SDNs have the ability to recognize specific DNA sequences and create DNA double-strand breaks (DSBs) within their target sites. Mutations are often introduced during the repair of DSBs. Directed DNA sequence modifications can occur when the SDNs are delivered along with homologous donor DNA; in these cases, the DSBs are repaired via the homologous recombination (HR) pathway. In plant cells, however, HR is the less favored repair pathway. The DSBs are typically repaired via non-homologous end-joining (NHEJ) pathways, which do not require the presence of homologous donor DNA and often lead to insertion and deletion (indel) mutations (Bétermier et al. 2014; Osakabe and Osakabe 2014; Vu et al. 2014).

ZFNs were first created in the 1990s (Kim et al. 1996; Porteus 2009) and the preferred means for gene editing when this study was initiated. The DNA-binding specificity of ZFNs is conferred by a Cys_2His_2 zinc-finger protein array, and the cleavage activity is provided by a *Fok*I endonuclease (Urnov et al. 2010; DeSouza 2012). Because the *Fok*I cleavage domain functions as a dimer, ZFNs cause site-specific DSBs. ZFNs have been used to mutagenize pre-integrated reporter genes and endogenous genes in several plant species, including *Arabidopsis thaliana* (*Arabidopsis*), *Nicotiana tabacum* (tobacco), *Petunia hybrida* (petunia), *Zea mays* (maize), *Glycine max* (soybean), *Malus domestica* (apple, cultivar Galaxy), and *Ficus carica* (fig, cultivar Kadota) (reviewed by Urnov et al. 2010; Carroll 2011; Petolino 2015; Weeks et al. 2016). Maeder et al. (2008) used one ZFN pair to target both of the tobacco acetolactate synthase genes (*SuRA* and *SuRB*); they observed single base pair deletions in 1.5 % of potential target alleles. Shukla et al. (2009) created a ZFN pair targeting the maize *INOSITOL 1, 3, 4, 5, 6-PENTAKISPHOSPHATE 2-KINASE* (*IPK1*) gene. The *ZFN* gene was driven by the maize ubiquitin-1 promoter (ZmUbi1). Transient expression in maize cells induced indel mutations in less than 0.05 % of the examined chromatids. Using a heat-inducible ZFN system, Osakabe et al. (2010) mutagenized the *ABA INSENSITIVE-4* (*ABI4*) gene in *Arabidopsis*; the frequency of indels ranged from 0.3 to 3 % among nine transgenic lines. The mutation rates reported by Zhang et al. (2010a) were 7 and 16 % in T_1 *Arabidopsis* plants, where estrogen-inducible ZFNs were used to target the *ALCOHOL DEHYDROGENASE1* (*ADH1*) and the *TRANSPARENT TESTA4* (*TT4*) genes. Curtin et al. (2011) used a similar estrogen-inducible ZFN system to target two *DICER-LIKE* (*DCL*) genes (*DCL4a* and *DCL4b*) in soybean; they found small insertions in two of the three recovered T_0 plants. Peer et al. (2015) reported the first use of ZFNs in tree species, where a heat-inducible ZFN expression system was adopted to repair a mutated *GUS* gene in two transgenic apple lines and three transgenic fig lines. They observed GUS expression in all of the five tested transgenic lines upon a 2.5-h heat treatment at 42 °C. In *Arabidopsis* (Osakabe et al. 2010; Zhang et al. 2010a) and tobacco (Maeder et al. 2008), biallelic mutations were identified at a rate of 10–30 % among the mutagenized population. These documented high rates of targeted mutagenesis and biallelic modification suggest that ZFNs could be a

promising tool to mutagenize essential floral genes and thus impart sexual sterility to tree species.

Two genes that are appealing targets for achieving floral sterility are *LEAFY* (*LFY*) and *AGAMOUS* (*AG*). These transcription factors are key regulators for floral initiation and floral organ identity (Causier et al. 2010; Moyroud et al. 2010; Galimba et al. 2012). *LFY* defines floral meristem identity. Most flowers in strong *lfy* mutants have been found to be sterile; these flowers open late and have abnormal morphologies, with a combination or mosaic of leaves, sepals, and carpels (Chandler 2012; Grandi et al. 2012; Mitsuda et al. 2006). *AG* is a C-function floral homeotic gene (Coen and Meyerowitz 1991). Its encoding protein regulates the differentiation of stamens and carpels and interacts with other genes to cause floral determinacy (Doerner 2001). Flowers in strong *ag* mutants usually undergo a conversion of reproductive floral organs to non-reproductive floral organs and have a "roselike" phenotype: the stamens are transformed to petals, and the central gynoecium is transformed to a sepal-/petal-looking internal flower (Parcy et al. 2002; Causier et al. 2010; Galimba et al. 2012). We have previously identified one ortholog of *LFY* (*PtLFY*) and two orthologs of *AG* (*PtAG1* and *PtAG2*) in *Populus trichocarpa* "Nisqually-1" (Brunner et al. 2000; Rottmann et al. 2000). The expression patterns of *PtLFY* and *PtAGs* indicate that they may have conserved functions during floral development in poplars, and the high similarity between the two *PtAG* genes (89 % amino acid identity: Brunner et al. 2000) should enable simultaneous targeting using a single ZFN pair.

Here we show that a heat-inducible ZFN system can mutagenize our selected floral genes in poplars at a rate of zero to 0.3 % (per explant per allele). We also report reduced shoot regeneration rate and transformation rate as a result of heat treatment and ZFN expression. These results suggest that the technology as tested is inefficient at producing biallelic mutations at the target loci, which would be essential for elimination of gene function.

Materials and methods

Plant materials

Two hybrid poplar clones, INRA 717-1B4 (female, *Populus tremula* × *P. alba*; hereafter referred to as clone 717) and INRA 353-38 (male, *P. tremula* × *P. tremuloides*; hereafter referred to as clone 353) that have been used extensively for transgenic studies (*e.g.*, Strauss et al. 2004; Zhang et al. 2010b), were used for *Agrobacterium*-mediated plant transformation. For both clones, plant material had been micropropagated in tissue culture for 7 years (hereafter referred to as 7-year-old clone 717 or 353 cultures) prior to initial transformation in 2012. In addition, clone 717 was re-established into sterile culture using field-grown material in 2012 (hereafter referred to as 1-year-old clone 717 cultures), which we used for transformation in 2013.

Sequencing of *PtLFY* and *PtAGs* in target poplars

Homologous sequences corresponding to our previously sequenced *PtLFY* (GenBank accession number U93196) and *PtAG*s (GenBank accession numbers AF052570 and AF052571) (Brunner et al. 2000; Rottmann et al. 2000) were identified from the *P. trichocarpa* genome sequence (version 3.0, available on the Phytozome website https://phytozome.jgi.doe.gov/pz/portal.html; *PtLFY*: Potri.015G106900, *PtAG1*: Potri.004G064300 and *PtAG2*: Potri.011G075800). The first exons of *PtLFY* (436 bp) and *PtAGs* (227 bp) were amplified using PCR from the genomic DNA (gDNA) of poplar clones 717 and 353. Several PCR products were sequenced from both ends to capture allelic differences or single-nucleotide substitutions (SNPs). Nucleotide sequence alignments against the *P. trichocarpa* "Nisqually-1" gene models revealed several regions in *PtLFY* (exon 1 and the adjacent intron) and *PtAG*s (the MADS box in exon 1), respectively (Figs. S1 and S2 and electronic supplementary appendix S1 and S2), with 100 % sequence identity; these regions were targeted for ZFN mutagenesis.

Creation of ZFNs

Collaborating with DOW AgroSciences, we created, validated, and selected four ZFN pairs for this study. Briefly, gDNA sequences of PtLFY and PtAGs were first scanned; potential ZFNs were then designed and evaluated for their activity in silico (Fig. S3a). In total, 64 ZFNs were predicted to have high activity. These ZFNs, each containing four- to six-finger zinc-finger proteins (ZFPs), were assembled into 17 PtLFY-

targeting ZFN pairs and 27 PtAGs-targeting ZFN pairs. The activity of ZFN pairs was evaluated in budding yeast according to Doyon et al. (2008) and in mouse Neuro 2A cells using Dual-Luciferase® Reporter Assay System (Promega). Two PtAGs-targeting ZFN pairs (namely ZFN800 and ZFN801; target sites shown in Fig. S1) and two PtLFY-targeting ZFN pairs (namely ZFN802 and ZFN803; target sites shown in Fig. S2) were selected due to their high activity in yeast and/or mouse cells (data not shown). Accordingly, four ZFN encoding fragments (2322–2493 bp; electronic supplementary appendix S3) were produced and cloned into four ZFN expression vectors (hereafter referred to as pZFNs) by DOW AgroSciences. Within each pZFN, the two ZFNs were joined by a 2A linker from the *Thosea asigna* virus that contains nuclear localization sequences (NLSs) known to function in poplar cells (Kohler et al. 2008; Shukla et al. 2009) (Fig. S3b).

Construct assembly

The *ZFN* encoding fragments were cloned into a modified pUC vector using the *Sfo*I and *Hind*III restriction sites in the pZFNs. Using the *Spe*I and *EcoR*V enzymes, each of the four ZFN pairs, together with the terminator of the Cauliflower Mosaic Virus 35S gene (35S t) in the pUC vector, were then cloned into the pART27 vector backbone behind the soybean (*G. max*) heat-inducible promoter *HSP6871* (hereafter referred to as HSP:ZFN constructs, Fig. 1). The jellyfish (*Aequorea victoria*) enhanced green fluorescent protein (eGFP) gene (electronic supplementary appendix S3), driven by the soybean (*G. max*) *Gmubi1500* promoter, was cloned into the vector using *EcoR*V and acted as a reporter to monitor transformation and chimerism. As a transformation control, we also created a control construct in the same pART27 vector backbone using the *Spe*I and *EcoR*V enzymes, in which the *HSP6871* promoter drove the expression of the *eGFP* gene (hereafter referred to as HSP:eGFP construct; Fig. 1).

Plant transformation and confirmation of transformants

The HSP:ZFN and HSP:eGFP constructs were transformed into *Agrobacterium tumefaciens* (*A. tumefaciens*) strain AGL1 using a freeze thaw method and then transformed into clones 717 and 353 using the method described by Filichkin et al. (2006). Kanamycin was used to select for transgenic tissue, and timentin was used to select against *A. tumefaciens*. The eGFP fluorescence was checked within 1 h after each treatment for HSP:eGFP co-cultivated explants or weekly for HSP:ZFN co-cultivated explants. All eGFP fluorescence was checked and recorded using the SMT1 Stereo-Microscope System (Tritech Research, Inc. Los Angeles, CA, USA) installed with the IC Capture (V2.2) software.

Polymerase chain reaction (PCR) was used to screen for and confirm transformants. Shoot tips from in vitro propagated poplars (2 months in root induction medium; height of ~10 cm) were harvested for gDNA extraction according to Crowley et al. (2003). gDNA quality and quantity were checked using a ND-1000 UV–Vis Spectrophotometer. All gDNA samples were diluted to 20 ng/µl before being used for molecular analyses. Due to the difficulties in designing efficient primers within the highly repetitive *ZFN* genes, primer pairs were designed to test the presence of the *eGFP* transgene (~750 bp) in the genome of the transgenic population. The locations of primers are indicated in Fig. 1; sequences are shown in Table S1.

Heat shock treatments

Using HSP:eGFP co-cultivated explants, we tested two heat shock methods for their efficiency in inducing transient transgene expression. For both poplar clones, explants from two independent transformation tests were tested under each of the two heat shock conditions. To minimize chimerism, we started heat treatments at the very beginning of the organogenesis stage (i.e., after 2 days of co-cultivation). In heat shock method 1 (HSM1), explants were incubated at 42 °C for 16 h twice, with a 32-h recovery at 22 °C between heat treatments. In heat shock method 2 (HSM2), explants were incubated at 42 °C for 3 h every day for 10 days, and explants were cultivated at 22 °C the rest of the time (Fig. S4). To monitor the expression of the *eGFP* gene in response to heat treatment, we recorded eGFP intensity (i.e., the presence of any visually detectable eGFP expression on an explant) after each heat treatment. Both HSMs were adopted in later experiments to trigger the expression of transgenes (i.e., *ZFN*s or *eGFP*).

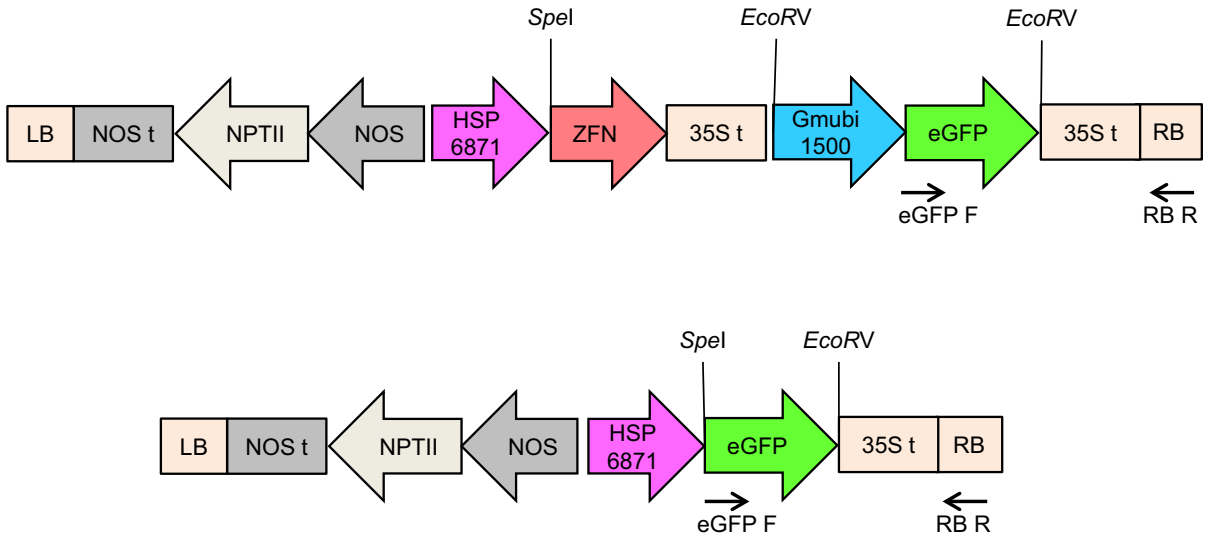

Fig. 1 T-DNA structure, and selected restriction enzyme and primer sites, for the HSP:ZFN (*above*) and HSP:eGFP (*below*) constructs within the pART27 binary vector backbone. The positions of primers used for PCR confirmation of transformation (eGFP F and RB R) are labeled. LB, left border; NOS t, terminator of nopaline synthase gene; NPTII, neomycin phosphotransferase gene; NOS, nopaline synthase promoter; HSP6871, heat shock promoter from soybean (*G. max*); ZFN, ZFN genes created by Dow AgroSciences; 35S t, terminator of cauliflower mosaic virus 35S gene; Gmubi1500, *G. max* polyubiquitin promoter; eGFP, enhanced green fluorescent protein gene from jellyfish (*Aequorea victoria*); RB, right border

Mutation screening and characterization

To screen for ZFN-induced mutations in the transgenic population, we performed high-resolution melting (HRM) analysis. HRM was performed on the StepOnePlus™ platform (Applied Biosystems, Foster City, CA, USA), and data were analyzed using the HRM Software V3.0.1(Applied Biosystems, Foster City, CA, USA), according to the manufacturer's protocol. We first evaluated the sensitivity of HRM analysis in detecting naturally occurring SNPs in *PtLFY* in clone 717 (electronic supplementary appendix S2). Briefly, PCR amplicons, with sizes of 84, 107, and 136 bp and containing zero, one and two SNP(s), respectively, were amplified from clone 717. Mixed gDNA samples from three independent isolations were used for HRM amplification and analysis to ensure the presence of the SNPs. Corresponding regions were also amplified from clone 353, in which no SNPs existed, and were used as references in HRM analysis. In screening for ZFN-induced mutations, short gDNA fragments covering ZFN targets were amplified (primers listed in Table S1; the sizes of the amplicons were 178 bp from *PtAG*s and 107 bp from *PtLFY*) and heated to 95 °C for denaturation. Three technical replicates were performed for each transgenic event. Amplification products showing changed melt curves and/or shifted T_m values (T_m, the temperature at which 50 % of the DNA duplexes are separated) were analyzed using agarose gel (3 %) electrophoresis, and also purified and cloned into the pCR™4-TOPO® TA Vector (Invitrogen) according to the manufacturer's protocol and transformed into chemically competent *Escherichia coli* (*E. coli*) cells. For each putative mutant event, 12–22 *E. coli* colonies were selected for plasmid extraction using the Zyppy™ Plasmid Miniprep Kit (Zymo Research, Orange, CA) and insert sequencing using Sanger methods.

Quantitative analysis

Shoot regeneration rate was calculated as the number of explants produced shoots per number of co-cultivated explants; similarly, transformation rate was the number of confirmed transgenic shoots per number of co-cultivated explants. Due to moderate microorganism contamination, several transformation tests that underwent HSM1 were excluded from calculations of shoot regeneration rate and transformation rate. These tests include two with the HSP:eGFP construct (one with clone 717 and one with clone 353), two with the HSP:ZFN800 construct (one with clone 717 and one

with clone 353), two with the HSP:ZFN801 construct (one with clone 717 and one with clone 353), and one with the HSP:ZFN803 construct in clone 353. ANOVA was used to examine whether shoot regeneration rate and transformation rate differed among constructs. Because we tested the HSP:ZFN800 and HSP:ZFN803 constructs in two sets of clone 717 in vitro shoot cultures that differed in age (see above), we calculated shoot regeneration rate and transformation rate separately for each set and checked for differences using ANOVA.

To compare the activity of ZFNs in different host systems, the activity scores in mouse cells and yeast (provided by Dow AgroSciences) were first normalized according to this equation:

$$Z_i = \frac{X_i - X_{\text{Min}}}{X_{\text{Max}} - X_{\text{Min}}},$$

where Z_i = normalized activity of ZFN pair i, X_i = observed activity of ZFN pair i, X_{Min} = minimum activity observed with all tested ZFN pairs, X_{Max} = maximum activity observed with all tested ZFN pairs.

Mutagenesis rate associated with each HSP:ZFN construct in poplars was also normalized using the same equation described above and used as the indicator of ZFN activity in poplars. Likewise, to examine the effect of ZFNs on the growth of host cells, concentrations of viable yeast were determined according to the OD_{600} readings after 24-h incubation (provided by Dow AgroSciences). Relative transformation rate of each HSP:ZFN construct in poplars was calculated by dividing the observed transformation rate by the rate obtained with the HSP:eGFP construct. The concentration of viable yeast and relative transformation rate were normalized and used as the indices of the effect of ZFNs on the viability of hosts.

To evaluate whether ZFN mutants were heterozygous (i.e., non-biallelic) or chimeras, we performed exact binomial tests with the null hypothesis that the frequency of observing a sequence was equal to 0.5. All statistical analyses were performed in R (version 3.1.2, 2014).

Results

Heat shock responses

To examine the activity of the *HSP6871* promoter and estimate the expression of *ZFN* genes upon heat treatment, we tested two heat shock conditions and calculated the percentage of explants with eGFP fluorescence. Clone 717 had an overall twofold higher rate of eGFP expression than did clone 353; both clones, however, had similar patterns of eGFP expression upon heat treatment (Fig. 2 and Table S2). With HSM1, the percentage of eGFP-expressing explants reached a maximum on day 4 (i.e., after the second 16-h heat treatment), with 37.4 % of 717 explants and 7.9 % of 353 explants showing eGFP signal. However, the number of eGFP-expressing explants shrunk rapidly without continued heat treatment and almost no explants showed eGFP signal on day 10 (i.e., after 6 days of recovery; Fig. S5). In contrast, eGFP expression induced by HSM2 was retained longer (Fig. S5); on average, there were 34.8 % of clone 717 explants and 15.4 % of clone 353 explants that maintained a detectable eGFP signal from day 3 to day 10.

In vitro plant regeneration was affected by heat shock treatments (Fig. S6 and Table S3). In both clones, more than 28.0 % of HSM1-treated explants developed shoots, while only 11.6–12.8 % of HSM2-treated explants did so. The decrease in shoot regeneration rate was statistically significant in clone 717 ($p = 0.03$) but fell just short of significance in clone 353 ($p = 0.08$). The transformation rate in clone 717 was 9.9 % under HSM1, but the percentage decreased significantly to 2.4 % when the explants were treated with HSM2 ($p = 0.04$). The transformation rates in clone 353 were 5.4 % under both heat treatment conditions.

Production of transgenic plants

To produce ZFN transgenic poplars, we co-cultivated over 2000 explants from each clone with each HSP:ZFN construct (Table S4) for a total of 21,698 explants. We obtained 2206 regenerated shoots, from which 391 were confirmed as transgenic by eGFP expression (Fig. 3) and/or PCR. No ZFN transgenic shoots were produced from HSM2, and we were unable to produce any confirmed HSP:ZFN802 transgenic events. PCR analysis of the *A. tumefaciens* strain (used for producing HSP:ZFN802 transgenic plants) failed to amplify a transgene product, despite its apparent successful transformation as judged by antibiotic resistance and colony PCR using standard methods. The other three HSP:ZFN constructs, HSP:ZFN800,

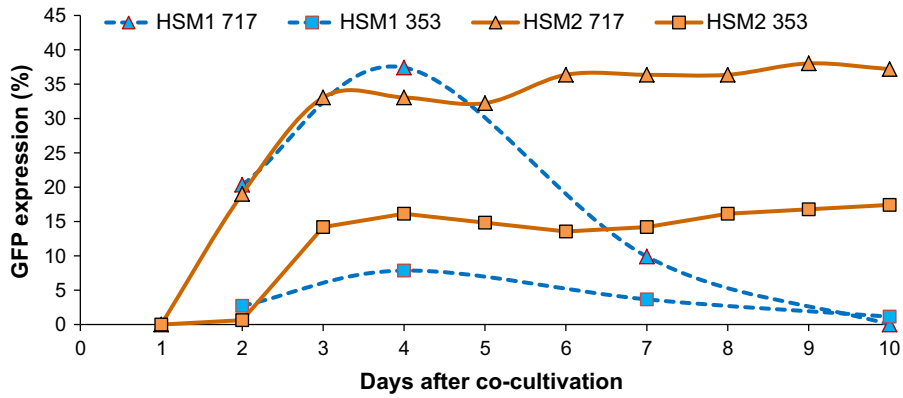

Fig. 2 Transient eGFP expression upon heat treatment in two poplar clones, 717 and 353. Percentages of explants with detectable eGFP fluorescence upon heat treatment are shown. For each clone and each heat shock method, 121–175 explants from two independent transformation tests were examined, therefore no error bars. HSM1, heat shock method 1; HSM2, heat shock method 2

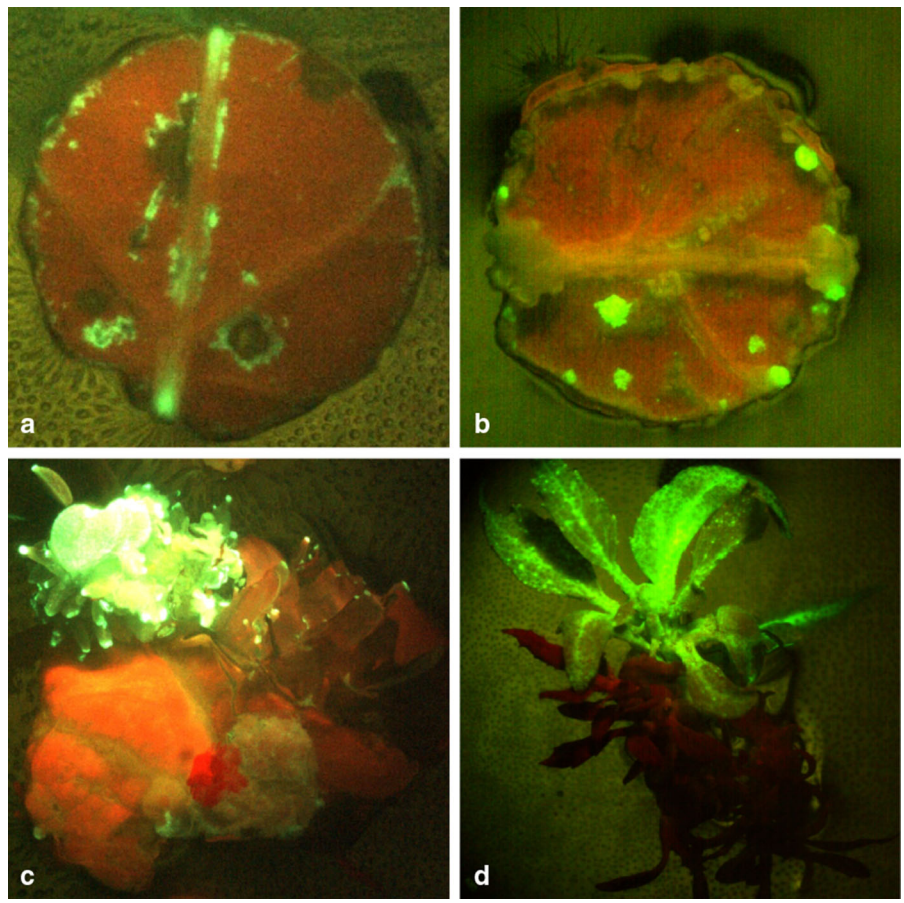

Fig. 3 Constitutive expression of eGFP in HSP:ZFN800 co-cultivated clone 717 explants. Photographs taken during organogenesis. **a** Two days on callus induction medium. **b** Three weeks on callus induction medium. **c** Three weeks on shoot regeneration medium. **d** Eight weeks on shoot regeneration medium

801, and 803, gave an overall shoot regeneration rate of 9.6 % and an overall transformation rate of 2.2 % (Fig. S7 and Table S4). These HSP:ZFN constructs, when compared with the HSP:eGFP construct (which showed an overall shoot regeneration rate of 28.7 % and an overall transformation rate of 7.7 % under HSM1; Table S3), showed significant (twofold to 11-fold) decreases in shoot regeneration rate and transformation rate under HSM1 (p values shown in Table S5). Comparison between yeast and poplars showed no obvious correlation in the effect of ZFNs on host viability (Table S6).

In an effort to enlarge the transgenic population so that higher numbers of mutated plants could be recovered and analyzed, we transformed over 2000 additional clone 717 explants with the HSP:ZFN800, HSP:ZFN803, and HSP:eGFP constructs (Table S7). Unlike early transformation tests, which were performed using shoot cultures that had been maintained in vitro for nearly 7 years (referred to as 7-year-old clone 717 cultures), these transformation tests used in vitro shoot cultures that had been established for only 1 year (referred to as 1-year-old clone 717 cultures; see methods). The shoot regeneration rates were 42.6 % for HSP:ZFN800, 40.9 % for HSP:ZFN803, and 83.1 % for HSP:eGFP, which showed significant (threefold to tenfold) increases compared with those obtained in our earlier transformation tests (Fig. S8a and Table S7). Transformation rates with the HSP:ZFN803 and HSP:eGFP constructs were 4.0 and 27 %, respectively, which also significantly increased compared to earlier tests (Fig. S8b and Table S7). In the case of the HSP:ZFN800 construct, although there was a twofold increase in the transformation rate, the change was not statistically significant ($p = 0.19$, Table S7). The 1-year-old 717 cultures, however, turned out to be heterozygous for one SNP in *PtAG1*, which was located in the left target region of ZFN800 (Fig. S9). Therefore, HSP:ZFN800 transformed 717 events obtained in later transformation tests (69 in total) were likely to be targeted only at the *PtAG2* locus but not at the *PtAG1* locus.

ZFN-induced mutagenesis

HRM analysis of naturally occurring SNPs in *PtLFY* was used to confirm its ability to detect 1- 2-bp changes within 110-bp amplicons (Fig. S10). To screen for mutations in ZFN transgenic poplars, we performed HRM to detect sequence changes near ZFN targets in *PtLFY*, *PtAG1*, or *PtAG2*. After analyzing 391 plants transformed with the HSP:ZFN800, 801, or 803 construct, only two transformed clone 353 events, namely event 0924-2 and event 1114-3 produced using the HSP:ZFN800 construct, were identified as putative mutants, as their *PtAG2* amplification products showed different melting curve shapes from wild-type (WT) amplification products (Fig. 4a).

To understand the nature of the mutations, we first performed agarose gel analysis of HRM products. Both WT and non-mutated ZFN transgenic events showed a single clear band on a 3 % gel, while the two putative mutants showed double bands. One band had a very similar mobility to the band in WT and non-mutated events, and the other band moved slower on the agarose gel (Fig. S11a), indicating a likely insertion.

We performed TOPO cloning to separate WT and mutated sequences into individual bacterial colonies. Among the 12 colonies transformed by event 1114-3, nine had WT sequences, and the other three had identical 7-bp deletions (Fig. 4b). Sequencing of 22 colonies produced from event 0924-2 revealed that nine colonies had WT sequence and 13 had deletions, with two different 7-bp deletions detected, one of which was more abundant than the other, being present in 12 of the 13 sequences (Fig. 4b). To confirm that the deletion identified in the single colony from event 0924-2 was not due to a sequencing error, we sequenced another 57 colonies that included gDNA samples isolated from shoot tips or mature leaves from two different shoots regenerated from the same event. Although at a low frequency, we were able to identify this same deletion in two additional samples (Table S8). Binomial tests suggested an equal amount of WT versus mutated sequences in both mutated events (Table S8). Therefore, both event 0924-2 and event 1114-3 were considered to be heterozygous mutations in further analysis. Considering all confirmed events and all four potential target alleles at the duplicated *PtAG* locus, the induced mutation rate for ZFN800 was 0.3 % per explant per allele. Despite this ZFN pair being the only active one in poplar, it did not show the highest mutagenesis activity in mouse- or yeast-based validation systems (Table S6).

As mentioned above, DNA amplification products from both mutated events contained duplexes with reduced gel mobility. However, we did not find sequences with insertions in either mutant. We

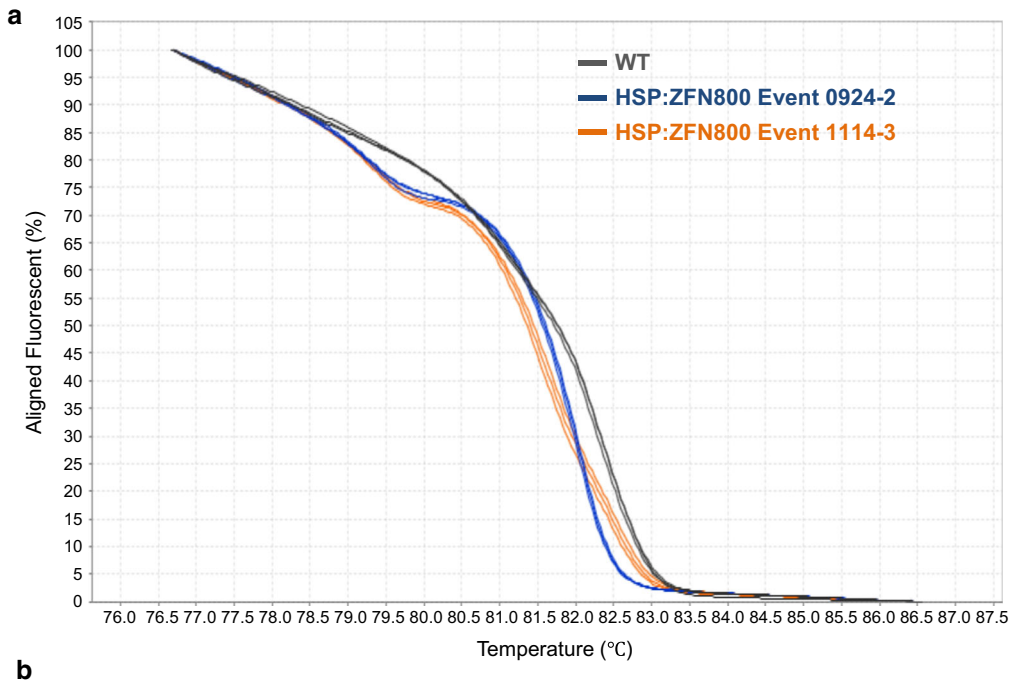

Fig. 4 Mutation detection and characterization in clone 353 HSP:ZFN800 transformants using HRM and sequencing. **a** The putative mutants, event 0924-2 and event 1114-3, showed different melt curves from wild type (WT). **b** Sequencing results revealed deletions in *PtAG*2 in the two mutants. Sequence of *PtAG*2 from WT 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 (see "Materials and methods")

therefore hypothesized that there were heteroduplexes formed during amplification and the secondary structure of the heteroduplexes affected their mobility on gel. To test this hypothesis, we performed PCR using bacteria colonies carrying WT sequence or the abundant type of deletion, and their mixture. Amplification products from the colony mixture indeed resembled double bands on our agarose gels, while mixing products from the WT- and deletion-carrying colonies did not give double bands (Fig. S11b).

Discussion

Under our test conditions, where ZFN expression was induced by heat shock at 42 °C for about 30 h during early organogenesis, we observed mutation rates ranging from zero to 0.3 % per explant per allele. This rate is within the range of previously reported frequencies, which varies from 0.05 to 16 % (reviewed above), but is at the lower end of published estimates. Much higher rates (up to 3 %) have been obtained with a similar system in *Arabidopsis*, where ZFN expression was induced by heat incubation at 40 °C for 90 min (Osakabe et al. 2010).

The low mutagenesis rates could have resulted from low activity of ZFN proteins or low expression of *ZFN* genes, or both. The activity of each ZFN pair used in this study was validated in mouse and yeast. ZFN800, which successfully induced mutagenesis in poplars, showed low-to-moderate activities in mouse and yeast. In contrast, ZFN802 and ZFN803, the most

effective pairs in yeast and mice, respectively, failed to mutagenize their targets in poplars in our experiments. The activity of ZFN801 appeared to the lowest in all three host systems. The disagreements between the two validation systems, and its weak relationship to activity in poplar, appear to make it impossible to choose highly effective ZFNs without testing in the target system.

Although three of the tested ZFN pairs (ZFN800, 801, and 803) produced transgenic plants, they depressed transformation rate significantly compared with the HSP:eGFP construct. Apart from potential deleterious effect of ZFNs, the large T-DNA size (due to the presence of the 2300–2500-bp ZFN encoding genes and the 1979-bp Gmubi1500 promoter) in the HSP:ZFN constructs could have led to reduced transformation efficiency. We also observed significant differences in transformation efficiency when using clone 717 in vitro cultures that differed in age. The low efficiency obtained with the 7-year-old cultures may result from tissue culture-specific somaclonal variation (Larkin and Scowcroft 1981), which happens at a low rate in *Populus* (Brunner et al. 2004) but has been reported in several hybrid clones and can lead to changes in leaf morphology and growth rate (Son et al. 1993; Gamburg and Voinikov 2013; Thakur and Ishii 2014). It is also possible that naturally occurring variations, similar to what we observed in *PtAG1*, led to different transformation competence between the two sets of clone 717 cultures.

ZFNs can cleave at undesired sites (Townsend et al. 2009; Gabriel et al. 2011; Pattanayak et al. 2011). Strong and continuous expression of the *ZFN* genes, especially the ones targeting *PtAG*s, may cause unexpected mutations, for example, in MADS genes other than the *PtAG*s. We adopted the heat-inducible system to limit the duration of the *ZFN* gene expression and therefore reduce potential off-target effects of ZFNs. Additionally, the heat shock treatments were started at the beginning of the organogenesis stage, which should help to minimize chimerism in regenerated poplars. As observed in other plant species, such as tobacco (Schmülling and Schell 1993) and rice (Hiei et al. 1994), chimeras can arise due to the regeneration of transformed and untransformed cells or the regeneration of independent insertion events. In our case, a chimera may contain wild-type or distinctly mutated copies of *PtLFY* or *PtAG*s due to the variation in *ZFN* expression over time. By expressing the *ZFN* genes mainly at early organogenesis via heat shock treatments, we were hoping to reduce the duration of ZFN expression and subsequent chimerism in induced mutations. However, heat treatments at 42 °C, especially prolonged treatments employed in HSM2, turned out to negatively affect in vitro regeneration of transgenic plants. While it is known that prolonged heat treatments can negatively impact plant performance, for example, stem elongation in tobacco (VanLoven et al. 1993; Bultynck et al. 1997), we had hoped that these plants would have achieved a high rate of gene targeting, as the ZFN expression would be induced for a longer period of time, which would help to offset reduced transformation rates.

Heat stress can also lead to reduced genome stability (Waterworth et al. 2011), but its effect on the NHEJ repair pathway and the subsequent mutation efficiency is unclear. In heat-stressed *Arabidopsis* plants, genes that encode Ku70 and Ku80—the key initiators of NHEJ, are down-regulated (Liu et al. 2008), whereas the rate of HR increases (Yao and Kovalchuk 2011). These observations suggest a predominant role of HR in repairing DSBs in response to heat stress. Additionally, *Arabidopsis* plants grown at suboptimal temperatures (either 4 or 32 °C) were found to contain proportionally less DNA compared with those grown at 22 °C (Boyko et al. 2005), a possible indicator of lower cell division (and thus of DNA replication), possibly limiting opportunities for DNA replication errors and subsequent NHEJ repair. The combined effects of a low NHEJ frequency and low regeneration rate from heat stress may have greatly constrained our production of indel mutations.

We found that both mutated events had deletions in only one allele of *PtAG2*; moreover, they had a WT copy *PtAG1* maintaining its full biological function. Therefore, it is unlikely that these events would produce sterile flowers. Given the observed transformation rate (2 %) and mutagenesis rate (0.3 % per explant per allele) in our study, we estimate that co-cultivation of at least 10,000 explants (i.e., two and half months of intensive transformation work) would be needed to produce one event with a biallelic mutation. Although this rate would be useful in a high value crop and trait, more efficient mutagenesis systems are likely to be needed for genetic containment purposes in forest trees (where a number of genotypes generally need to be produced, tested, and

deployed commercially). This could be done through increasing activity of ZFNs (*e.g.*, redesigning ZFNs with different platforms; reviewed by Urnov et al. 2010; Voytas 2013) or improving the specificity of ZFNs (as demonstrated by Miller et al. 2007; Händel et al. 2009). Alternative SDNs, for example, CRISPR/Cas9, has been used to successfully produce a high rate of biallelic mutations in *LFY* in *Populus* (Elorriaga et al. 2015); it therefore appears to be a superior tool for imparting sterility. Off-target effects of SDNs also need to be evaluated to avoid unpredictable effects on plant growth; the low rate of transformation we observed with ZFNs suggests that this might also be problematic with ZFNs.

Acknowledgments We thank many undergraduate students for their help, including Ruby Chen, Tommy Nguyen, and Samantha S. Colby for helping with plant transformation, Hui Yan for preparing medium for plant tissue culture, and Jessica A. Greer, and Katherine I. Walgrave for assisting in genomic DNA isolation. We thank Dow AgroSciences for designing, producing, and selling us the *ZFN* constructs; Jim Thompson of USDA-ARS for helping with vector development; and John Finer of Ohio State for providing the *eGFP* marker gene. This project is supported by USDA Grants No. 2010-33522-21736 (Biotechnology Risk Assessment) and 2011-68005-30407 (Biofuels CAP) from the National Institute of Food and Agriculture. We also thank industrial members of the Tree Biosafety and Genomics Research Cooperative (TBGRC) at OSU for indirect support.

References

Ain QU, Chung JY, Kim YH (2015) Current and future delivery systems for engineered nucleases: ZFN, TALEN and RGEN. J Controlled Release 205:120–127

Belhaj K, Chaparro-Garcia A, Kamoun S, Nekrasov V (2013) Plant genome editing made easy: targeted mutagenesis in model and crop plants using the CRISPR/Cas system. Plant Methods 9(1):39

Bennett AB, Chi-Ham C, Barrows G, Sexton S, Zilberman D (2013) Agricultural biotechnology: economics, environment, ethics, and the future. Annu Rev Environ Resour 38(1):249–279

Bétermier M, Bertrand P, Lopez BS (2014) Is non-homologous end-joining really an inherently error-prone process? PLoS Genet 10(1):e1004086

Boyko A, Filkowski J, Kovalchuk I (2005) Homologous recombination in plants is temperature and day-length dependent. Mutat Res, Fundam Mol Mech Mutagen 572(1):73–83

Brunner AM, Rottmann WH, Sheppard LA, KrutovskiiK DiFazio SP, Leonardi S, Strauss SH (2000) Structure and expression of duplicate AGAMOUS orthologues in poplar. Plant Mol Biol 44(5):619–634

Brunner AM, Busov VB, Strauss SH (2004) Poplar genome sequence: functional genomics in an ecologically dominant plant species. Trends Plant Sci 9(1):49–56

Brunner AM, Li J, DiFazio SP, Shevchenko O, Montgomery BE, Mohamed R, Wei H, Ma C, Elias AA, VanWormer K, Strauss SH (2007) Genetic containment of forest plantations. Tree Genet Genomes 3(2):75–100

Bultynck L, Geuns JM, VanGinkel G, Caubergs RJ (1997) Properties of plasma membranes of Phsp 70-Ipt transformed tobacco (*Nicotiana Tabacum*). Phytochemistry 45(7):1337–1341

Carroll D (2011) Genome engineering with zinc-finger nucleases. Genetics 188(4):773–782

Causier B, Schwarz-Sommer Z, Davies B (2010) Floral organ identity: 20 years of ABCs. Semin Cell Dev Biol 21(1):73–79

Chandler JW (2012) Floral meristem initiation and emergence in plants. Cell Mol Life Sci 69(22):3807–3818

Coen ES, Meyerowitz EM (1991) The war of the whorls: genetic interactions controlling flower development. Nature 353(6339):31–37

Crowley TM, Muralitharan MS, Stevenson TW (2003) Isolating conifer DNA: a superior polysaccharide elimination method. Plant Mol Biol Rep 21(1):97

Curtin SJ, Zhang F, Sander JD, Haun WJ, Starker C, Baltes NJ, Reyon D, Dahlborg EJ, Goodwin MJ, Coffman AP, Dobbs D, Joung JK, Voytas DF, Stupar RM (2011) Targeted mutagenesis of duplicated genes in soybean with zinc-finger nucleases. Plant Physiol 156(2):466–473

DeSouza N (2012) Primer: genome editing with engineered nucleases. Nat Methods 9(1):27

DiFazio SP, Leonardi S, Slavov GT, Garman SL, Adams WT, Strauss SH (2012) Gene flow and simulation of transgene dispersal from hybrid poplar plantations. New Phytol 193(4):903–915

Doerner P (2001) Plant meristems: a ménage à trois to end it all. Curr Biol 11(19):R785–R787

Doyon Y, McCammon JM, Miller JC, Faraji F, Ngo C, Katibah GE, Amora R, Hocking TD, Zhang L, Rebar EJ, Gregory PD, Urnov FD, Amacher SL (2008) Heritable targeted gene disruption in zebrafish using designed zinc-finger nucleases. Nat Biotechnol 26(6):702–708

Dunwell JM (2014) Genetically modified (GM) crops: European and transatlantic divisions. Mol Plant Pathol 15(2):119–121

Elorriaga E, Meilan R, Ma C, Skinner JS, Etherington E, Brunner AM, Strauss SH (2014) A tapetal ablation transgene induces stable male sterility and slows field growth in *Populus*. Tree Genet Genomes 10(6):1583–1593

Elorriaga E, Klocko A, Ma C, Strauss SH (2015) *CRISPR-Cas nuclease mutagenesis for genetic containment of genetically engineered forest trees*. Minneapolis, MN: American Society of Plant Biologists Meeting. http://plantbiology.aspb.org/wp-content/uploads/2015/07/FINAL-Poster-Abstracts-w.-Cover-Page-.pdf

Filichkin SA, Meilan R, Busov VB, Ma C, Brunner AM, Strauss SH (2006) Alcohol-inducible gene expression in transgenic Populus. Plant Cell Rep 25(7):660–667

Gabriel R, Lombardo A, Arens A, Miller JC, Genovese P, Kaeppel C, Nowrouzi A, Bartholomae CC, Wang J, Friedman G, Holmes MC, Gregory PD, Glimm H, Schmidt

M, Naldini L, VonKalle C (2011) An unbiased genome-wide analysis of zinc-finger nuclease specificity. Nat Biotechnol 29(9):816–823

Galimba KD, Tolkin TR, Sullivan AM, Melzer R, Theißen G, DiStilio VS (2012) Loss of deeply conserved C-Class floral homeotic gene function and C- and E-class protein interaction in a double-flowered ranunculid mutant. Proc Natl Acad Sci USA 109(34):E2267–E2275

Gamburg KZ, Voinikov VK (2013) Somaclonal variations as a mean for obtaining regenerants with different growth rates in poplar (*Populus* × *berolinensis* Dipp.). Nat Sci 5(5):599–607

Grandi V, Gregis V, Kater MM (2012) Uncovering genetic and molecular interactions among floral meristem identity genes in *Arabidopsis thaliana*. Plant J 69(5):881–893

Händel EM, Alwin S, Cathomen T (2009) Expanding or restricting the target site repertoire of zinc-finger nucleases: the inter-domain linker as a major determinant of target site selectivity. Mol Ther 17(1):104–111

Hiei Y, Ohta S, Komari T, Kumashiro T (1994) Efficient transformation of rice (*Oryza sativa* L.) mediated by Agrobacterium and sequence analysis of the boundaries of the T-DNA. Plant J 6(2):271–282

Hoenicka H, Lehnhardt D, Polak O, Fladung M (2012) Early flowering and genetic containment studies in transgenic poplar. iForest Biogeosci For 5(3):138–146

Kim YG, Cha J, Chandrasegaran S (1996) Hybrid restriction enzymes: zinc finger fusions to Fok I cleavage domain. Proc Natl Acad Sci 93(3):1156–1160

Kohler A, Rinaldi C, Duplessis S, Baucher M, Geelen D, Duchaussoy F, Meyers BC, Boerjan W, Martin F (2008) Genome-wide identification of NBS resistance genes in *Populus trichocarpa*. Plant Mol Biol 66(6):619–636

Larkin PJ, Scowcroft WR (1981) Somaclonal variation—a novel source of variability from cell cultures for plant improvement. Theor Appl Genet 60(4):197–214

Li Y, Peng Y, Hallerman EM, Wu K (2014) Biosafety management and commercial use of genetically modified crops in China. Plant Cell Rep 33(4):565–573

Liu PF, Wang YK, Chang WC, Chang HY, Pan RL (2008) Regulation of *Arabidopsis thaliana* Ku genes at different developmental stages under heat stress. Biochim Biophys Acta Gene Regul Mech 1779(6–7):402–407

Maeder ML, Thibodeau-Beganny S, Osiak A, Wright DA, Anthony RM, Eichtinger M, Jiang T, Foley JE, Winfrey RJ, Townsend JA, Unger-Wallace E, Sander JD, Müller-Lerch F, Fu F, Pearlberg J, Göbel C, Dassie JP, Pruett-Miller SM, Porteus MH, Sgroi DC, Iafrate AJ, Dobbs D, McCray PB Jr, Cathomen T, Voytas DF, Joung JK (2008) Rapid "open-source" engineering of customized zinc-finger nucleases for highly efficient gene modification. Mol Cell 31(2):294–301

Miller JC, Holmes MC, Wang J, Guschin DY, Lee YL, Rupniewski I, Beausejour CM, Waite AJ, Wang NS, Kim KA, Gregory PD, Pabo CO, Rebar EJ (2007) An improved zinc-finger nuclease architecture for highly specific genome editing. Nat Biotechnol 25(7):778–785

Mitsuda N, Hiratsu K, Todaka D, Nakashima K, Yamaguchi-Shinozaki K, Ohme-Takagi M (2006) Efficient production of male and female sterile plants by expression of a chimeric repressor in arabidopsis and rice. Plant Biotechnol J 4(3):325–332

Moriguchi Y, Ueno S, Saito M, Higuchi Y, Miyajima D, Itoo S, Tsumura Y (2014) A simple allele-specific PCR marker for identifying male-sterile trees: towards DNA marker-assisted selection in the Cryptomeria japonica breeding program. Tree Genet Genomes 10(4):1069–1077

Moyroud E, Kusters E, Monniaux M, Koes R, Parcy F (2010) LEAFY blossoms. Trends Plant Sci 15(6):346–352

Osakabe Y, Osakabe K (2014) Genome editing with engineered nucleases in plants. Plant Cell Physiol pcu170

Osakabe K, Osakabe Y, Toki S (2010) Site-directed mutagenesis in Arabidopsis using custom-designed zinc finger nucleases. Proc Natl Acad Sci 107(26):12034–12039

Parcy F, Bomblies K, Weigel D (2002) Interaction of LEAFY, AGAMOUS and TERMINAL FLOWER1 in maintaining floral meristem identity in Arabidopsis. Development 129(10):2519–2527

Pattanayak V, Ramirez CL, Joung JK, Liu DR (2011) Revealing off-target cleavage specificities of zinc-finger nucleases by in vitro selection. Nat Methods 8(9):765–770

Peer R, Rivlin G, Golobovitch S, Lapidot M, Gal-On A, Vainstein A, Tzfira T, Flaishman MA (2015) Targeted mutagenesis using zinc-finger nucleases in perennial fruit trees. Planta 241(4):941–951

Petolino JF (2015) Genome editing in plants via designed zinc finger nucleases. In Vitro Cell Dev Biol Plant 51(1):1–8

Porteus MH (2009) Plant biotechnology: zinc fingers on target. Nature 459(7245):337–338

Rottmann WH, Meilan R, Sheppard LA, Brunner AM, Skinner JS, Ma C, Cheng S, Jouanin L, Pilate G, Strauss SH (2000) Diverse effects of overexpression of LEAFY and PTLF, a Poplar (Populus) homolog of LEAFY/FLORICAULA, in transgenic poplar and Arabidopsis. Plant J 22(3):235–245

Schmülling T, Schell J (1993) Transgenic tobacco plants regenerated from leaf disks can be periclinal chimeras. Plant Mol Biol 21(4):705–708

Shukla VK, Doyon Y, Miller JC, DeKelver RC, Moehle EA, Worden SE, Mitchell JC, Arnold NL, Gopalan S, Meng X, Choi VM, Rock JM, Wu YY, Katibah GE, Zhifang G, McCaskill D, Simpson MA, Blakeslee B, Greenwalt SA, Butler HJ, Hinkley SJ, Zhang L, Rebar EJ, Gregory PD, Urnov FD (2009) Precise genome modification in the crop species *Zea mays* using zinc-finger nucleases. Nature 459(7245):437–441

Son SH, Moon HK, Hall RB (1993) Somaclonal variation in plants regenerated from callus culture of hybrid aspen (*Populus alba* L. × *P. Grandidentata* Michx.). Plant Sci 90(1):89–94

Stewart CN Jr, Halfhill MD, Warwick SI (2003) Transgene introgression from genetically modified crops to their wild relatives. Nat Rev Genet 4(10):806–817

Strauss SH, Rottmann WH, Brunner AM, Sheppard LA (1995) Genetic engineering of reproductive sterility in forest trees. Mol Breeding 1(1):5–26

Strauss SH, Brunner AM, Busov VB, Ma C, Meilan R (2004) Ten lessons from 15 years of transgenic Populus research. Forestry 77(5):455–465

Strauss SH, Schmitt M, Sedjo R (2009) Forest scientist views of regulatory obstacles to research and development of transgenic forest biotechnology. J Forest 107(7): 350–357

Strauss SH, Costanza A, Séguin A (2015) Genetically engineered trees: paralysis from good intentions. Science 349(6250):794–795

Thakur RC, Ishii K (2014) Detection and fingerprinting of narrow-leaf mutants in micro-propagated hybrid poplar (*Populus sieboldii* × *P. grandidentata*) using random amplified polymorphic DNA. Int J Farm Sci 2(1):79–84

Townsend JA, Wright DA, Winfrey RJ, Fu F, Maeder ML, Joung JK, Voytas DF (2009) High-frequency modification of plant genes using engineered zinc-finger nucleases. Nature 459(7245):442–445

Urnov FD, Rebar EJ, Holmes MC, Zhang HS, Gregory PD (2010) Genome editing with engineered zinc finger nucleases. Nat Rev Genet 11(9):636–646

VanLoven K, Beinsberger SE, Valcke RL, VanOnckelen HA, Clijsters HM (1993) Morphometric analysis of the growth of Phsp70-Ipt transgenic tobacco plants. J Exp Bot 44(11):1671–1678

Vining KJ, Contreras RN, Ranik M, Strauss SH (2012) Genetic methods for mitigating invasiveness of woody ornamental plants: research needs and opportunities. HortScience 47(9):1210–1216

Viswanath V, Albrectsen BR, Strauss SH (2012) Global regulatory burden for field testing of genetically modified trees. Tree Genet Genomes 8(2):221–226

Voytas DF (2013) Plant genome engineering with sequence-specific nucleases. Annu Rev Plant Biol 64(1):327–350

Vu GT, Cao HX, Watanabe K, Hensel G, Blattner FR, Kumlehn J, Schubert I (2014) Repair of site-specific DNA double-strand breaks in barley occurs via diverse pathways primarily involving the sister chromatid. Plant Cell 26(5): 2156–2167

Waterworth WM, Drury GE, Bray CM, West CE (2011) Repairing breaks in the plant genome: the importance of keeping it together. New Phytol 192(4):805–822

Weeks DP, Spalding MH, Yang B (2016) Use of designer nucleases for targeted gene and genome editing in plants. Plant Biotechnol J 14(2):483–495

Yao Y, Kovalchuk I (2011) Abiotic stress leads to somatic and heritable changes in homologous recombination frequency, point mutation frequency and microsatellite stability in Arabidopsis plants. Mutat Res, Fundam Mol Mech Mutagen 707(1):61–66

Zhang F, Maeder ML, Unger-Wallace E, Hoshaw JP, Reyon D, Christian M, Li X, Pierick CJ, Dobbs D, Peterson T, Joung JK, Voytas DF (2010a) High frequency targeted mutagenesis in *Arabidopsis thaliana* using zinc finger nucleases. Proc Natl Acad Sci 107(26):12028–12033

Zhang H, Harry DE, Ma C, Yuceer C, Hsu CY, Vikram V, Shevchenko O, Etherington E, Strauss SH (2010b) Precocious flowering in trees: the FLOWERING LOCUS T gene as a research and breeding tool in Populus. J Exp Bot 61(10):2549–2560

Zhang C, Norris-Caneda KH, Rottmann WH, Gulledge JE, Chang S, Kwan BYH, Thomas AM, Mandel LC, Kothera RT, Victor AD, Pearson L, Hinchee MAW (2012) Control of pollen-mediated gene flow in transgenic trees. Plant Physiol 159(4):1319–1334