Plant Biotechnology Journal Open Access

*Plant Biotechnology Journal* (2021) **19**, pp. 23–25 doi: 10.1111/pbi.13451

**Brief Communication**

# CRISPR/Cas9-mediated single and biallelic knockout of poplar *STERILE APETALA* (*PopSAP*) leads to complete reproductive sterility

Abdul Azeez and Victor Busov

*College of Forest Resources and Environmental Science, Michigan Technological University, Houghton, MI, USA*

*Received 22 April 2020;*
*revised 17 June 2020;*
*accepted 2 July 2020.*
*Correspondence* (Tel 906 487 1728; fax 906 487 2915; email vbusov@mtu.edu)

**Keywords:** CRISPR, transgene containment, sterility, poplar, gene editing, heat-inducible.

The spread of highly domesticated, exotic, or genetically modified organisms into wild and feral populations and/or their genes beyond the boundaries of plantations can negatively, irreversibly impact the health of native species and pose significant management problems in human-dominated ecosystems (Snow *et al*., 2005). Therefore, it is imperative to minimize or, if possible, completely avert the risks associated with such spread. Containment refers to approaches used to prevent the spread of such organisms (Brunner *et al*., 2007).

Containment approaches were first inspired in the early 1990s by the demonstration of genetically engineered male and female sterility (Mariani *et al*., 1990). Reproductive sterility effectively minimizes or voids any risk of spreading genes/transgenes into the wild by means of sexual reproduction by significantly reducing or preventing the generation of gametes. Inducing floral sterility is a feasible and relevant approach, particularly in trees. Many major tree crops, including poplar, are clonally propagated with rooted cuttings, and thus, a lack of seeds does not interfere with their propagation. Delaying or eliminating flowering is beneficial since this channels more assimilates to vegetative growth, the primary product in the forest plantation (Brunner *et al*., 2007).

Two major approaches to alter sexual reproduction have been tested. The first, known as floral ablation, involves the expression of toxic genes under a floral-predominant promoter. The expression of toxins in floral tissues either renders these tissues nonfunctional or destroys them. A major problem with floral ablation has been the ‘leaky’ expression of the toxic genes outside flower tissues, which significantly compromises vegetative growth (Wei *et al*., 2006). The second approach, which we employ in this study, involves the modification of genes that affect normal reproductive development or interfere with the transition to reproductive growth.

*STERILE APETALA* (*SAP*) was first discovered in *Arabidopsis thaliana* as the result of a SAP loss-of-function mutation that caused complete male and female sterility (Byzova *et al*., 1999). More recently, work in *A. thaliana* and our work in poplar showed that, in addition to having a role in reproductive development, SAP is also involved in vegetative growth, particularly in the regulation of leaf size (Li *et al*., 2018; Yordanov *et al*., 2017). Because of the similar roles of SAP in the regulation of vegetative growth in *A. thaliana* and poplar, we hypothesized that poplar *SAP* (*PopSAP*) knockout will lead to sterility in poplar as in *A. thaliana*.

Although long generation cycles are one of the greatest impediments to testing sterility technologies in poplar, this can be overcome by using inducible expression of *FT* (*FLOWERING LOCUS T*) orthologs (Zhang *et al*., 2010). First, we improved this inducible system to make it more suitable for testing sterility approaches in poplar (Azeez and Busov, 2019). After determining that the inconsistent, slow flowering was caused by low levels of *FT* induction brought about by the heat inductive system, we developed a more efficient method employing repeated cycles of heat induction followed by resting periods for recovery from heat shock (Azeez and Busov, 2019). This method was successfully tested in both male (353-FT, *Populus tremula x tremuloides*) and female (717-FT, *Populus tremula x alba*) poplar genotypes. Next, we utilized the modified induction method to test the efficiency of *PopSAP* knockout as a sterility technology. CRISPR/Cas9 was used to knockout *PopSAP* in both the male (353-FT) and female (717-FT) genotypes. The target site was selected based on location in the gene and GC content using Aspen DB (http://aspendb.uga.edu/index.php/databases/spta-717-genome; Figure 1a). The specificity of the sgRNA was also checked by BLAST searches in the aspen (http://popgenie.org/) and 717 genome databases (http://aspendb.uga.edu/index.php/databases/spta-717-genome). The expression cassette was assembled in a shuttle vector containing the *A. thaliana* AtU3d promoter and then transferred into the *pYLCRISPR/Cas9Pubi-H* binary vector (Ma *et al*., 2015). The construct was sequence-verified, transformed into *Agrobacterium* strain C58, and finally transformed into the male (353-FT) and female (717-FT) genotypes using leaf tissues. We produced numerous transgenic plants and sequenced more than 20 independent lines to identify knockouts of the *PopSAP* gene. We identified 10 lines with frameshift indels in one allele (Figure 1b, c) in both male and female genotypes but only one independent line (18-1/A) in the female genotype carrying frameshift mutations in both alleles (Figure 1b). This single line had significantly impaired growth (Figure 1d, f), suggesting that biallelic loss-of-function mutations in the *PopSAP* gene are difficult to recover likely due to impaired growth, problems with regeneration or combinations of these. Since *PopSAP* is a single gene in poplar and other genomes (Yordanov *et al*., 2017), there are no redundant copies of the gene; therefore, knocking out *PopSAP* likely significantly impairs growth and development. For this reason, we proceeded to characterize plants carrying heterozygous knockouts (only one allele mutated) and the single line carrying the biallelic mutations.

We selected six independent lines carrying different *PopSAP* knockout mutations for further analysis, including three

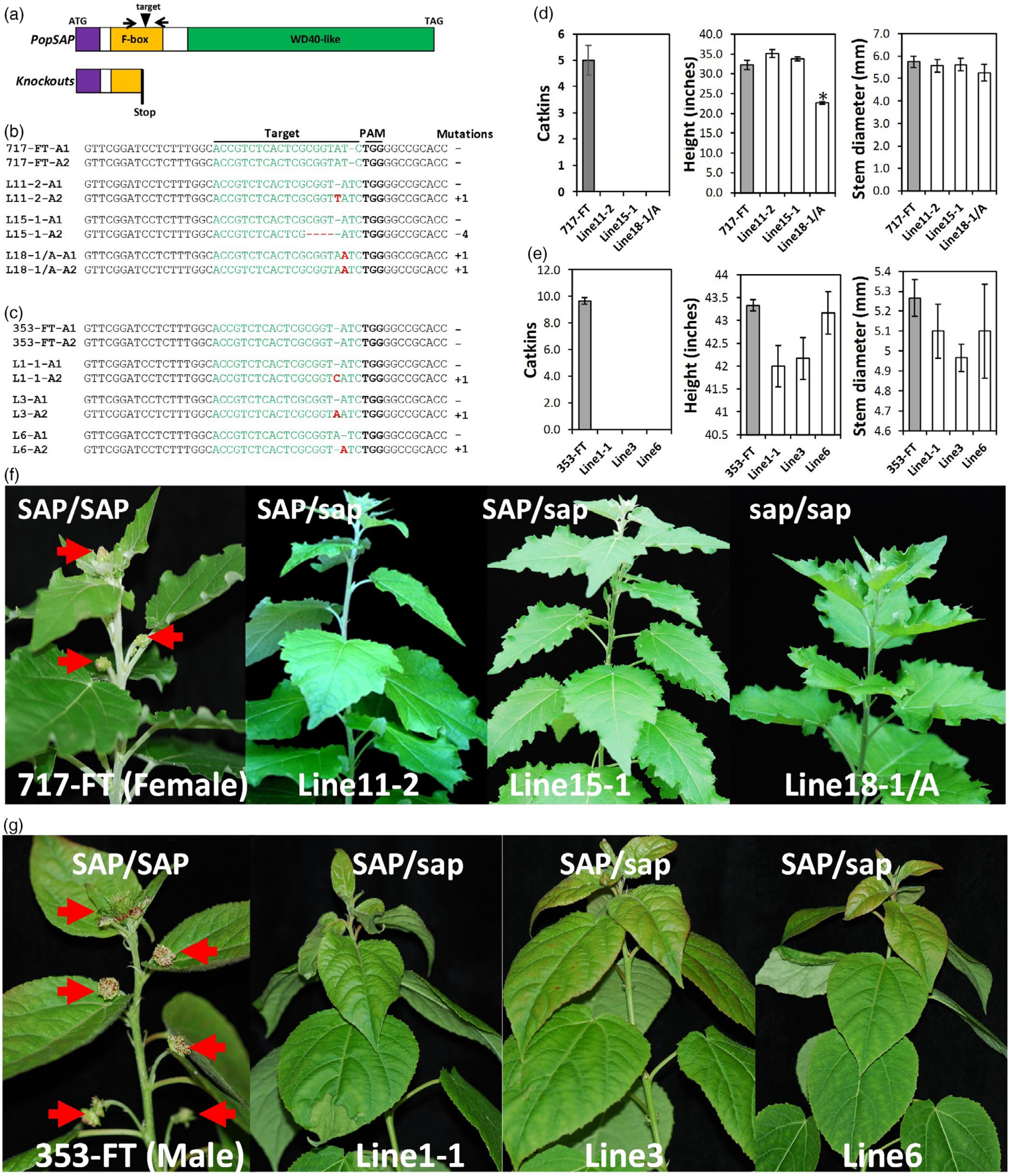

**Figure 1** Reproductive and vegetative development of different CRISPR/Cas9 *PopSAP* knockout lines in female 717-FT and male 353-FT genotypes. (a) Schematic of sgRNA selected to target coding sequences of *PopSAP*. The proximal site was selected for deletion of the functional domain (WD40-like). (b, c) Mutations in selected *PopSAP* CRISPR/Cas9 lines in the female 717-FT and male 353-FT genotypes, respectively. Protospace adjacent motif (PAM) and guide RNA (gRNA) target site (Target) are indicated on top. Deletions are marked with red dashes, and insertions are indicated with red letters. The numbers on the right indicate mutation type and the number of mutated nucleotides as follows: insertion (+), deletion (−), whereas (−) without a number indicates no mutation. A1 and A2 indicate allele 1 and allele 2. (d, e) Number of catkins, height and stem diameter of control and CRISPR/Cas9 lines of 717-FT and 353-FT genotypes, respectively. (f, g) 717-FT and 353-FT control plants produce catkins after heat induction whereas no catkins are observed in the respective CRISPR/Cas9 lines. Red arrows indicate growing catkins in control plants. Lines with a knockout mutation in one allele are indicated as *SAP/sap* while the line with a homozygous biallelic knockout is indicated as *sap/sap*. At least 6 ramets of the 353-FT and 717-FT control and CRISPR/Cas9 lines were used in these analyses. '*' indicates statistical significance at $P < 0.05$ compared to 717-FT control plants as determined by Student's *t*-test.

heterozygous lines in the male (353-FT) genotype, two heterozygous lines in the female (717-FT) genotype and the single female line with biallelic homozygous mutations (Line18-1/A). We then used our improved flower induction system to test the effect of these mutations on flower initiation. In contrast to control plants, the heterozygous knockouts in both male and female genotypes caused complete sterility with no initiation of inflorescences; this was also true for the biallelic knockout in the female genotype (Figure 1d-g). These results differ from *A. thaliana*, where flowers were observed but were sterile (Byzova *et al*., 1999). These differences are likely attributable to key differences in inflorescence development in the two species. Poplar initiates inflorescence development nearly a year before they fully develop, but in *A. thaliana* this occurs within the same year.

In *A. thaliana* and poplar (Yordanov *et al*., 2017), SAP regulates vegetative growth and development. For this reason, we determined whether the mutations in *PopSAP* affect vegetative growth. Indeed, in the female 717-FT genotype, the only plant carrying biallelic *PopSAP* loss-of-function mutations was significantly shorter than control plants (Figure 1d, f). In the male 353-FT genotype, some heterozygous *PopSAP* knockouts showed an appreciable reduction in growth (height), though this was not statistically significant (Figure 1e, g). Therefore, while heterozygous and biallelic knockouts of *PopSAP* likely affect vegetative growth, these effects differ depending on the clone used as well as heterozygous vs. biallelic knockout.

Long-term field testing is necessary to more reliably assess the effect of observed changes in vegetative growth. Studies in *A. thaliana* are beginning to clarify the role of SAP in vegetative growth. These studies demonstrate that by modifying SAP target genes it is possible to mitigate the effects of SAP knockout on vegetative growth; additional studies are necessary to ensure that sterility phenotypes are not compromised under these conditions.

Our results indicate that SAP is a promising target for developing sterility-based containment technologies in poplar. Further testing is needed, particularly under field conditions, to validate the robustness of the system and its effects on biomass accumulation.

## Acknowledgements

This project was supported by Biotechnology Risk Assessment Grant Program Competitive Grant no. 2016-33522-25626 from the U.S.D.A. We thank Galina Agapova and Naomi Ojala for propagation and care of the transgenic lines.

## Conflict of interest

The authors declare no financial conflict of interest.

## Author contributions

V.B. conceived the research. A.A. designed and executed the experiments. Both authors wrote the article.

## References

Azeez, A. and Busov, V.B. (2019) Improved heat FT induction ieads to earlier and more prolific flowering in poplar. *J. Botanical Res.* **1**, 15–18.

Brunner, A.M., Li, J., DiFazio, S.P., Shevchenko, O., Montgomery, B.E., Mohamed, R., Wei, H. *et al*. (2007) Genetic containment of forest plantations. *Tree Genet. Genomes*, **3**, 75–100.

Byzova, M.V., Franken, J., Aarts, M.G., de Almeida-Engler, J., Engler, G., Mariani, C., Van Lookeren Campagne, M.M. *et al*. (1999) Arabidopsis STERILE APETALA, a multifunctional gene regulating inflorescence, flower, and ovule development. *Genes Develop.* **13**, 1002–1014.

Li, N., Liu, Z., Wang, Z., Ru, L., Gonzalez, N., Baekelandt, A., Pauwels, L. *et al.* (2018) STERILE APETALA modulates the stability of a repressor protein complex to control organ size in Arabidopsis thaliana. *Plos Genet.* **14**, e1007218.

Ma, X., Zhang, Q., Zhu, Q., Liu, W., Chen, Y., Qiu, R., Wang, B. *et al*. (2015) A Robust CRISPR/Cas9 system for convenient, high-efficiency multiplex genome editing in monocot and dicot plants. *Mol. Plant*, **8**, 1274–1284.

Mariani, C., Debeuckeleer, M., Truettner, J., Leemans, J. and Goldberg, R.B. (1990) Induction of male-sterility in plants by a chimeric ribonuclease gene. *Nature*, **347**, 737–741.

Snow, A.A., Andow, D.A., Gepts, P., Hallerman, E.M., Power, A., Tiedje, J.M. and Wolfenbarger, L.L. (2005) Genetically engineered organisms and the environment: Current status and recommendations. *Ecol. Appl.* **15**, 377–404.

Wei, H., Meilan, R., Brunner, A.M., Skinner, J.S., Ma, C., Gandhi, H.T. and Strauss, S.H. (2006) Field trial detects incomplete barstar attenuation of vegetative cytotoxicity in Populus trees containing a poplar LEAFY promoter: barnase sterility transgene. *Mol. Breed.* **19**, 69–85.

Yordanov, Y.S., Ma, C., Yordanova, E., Meilan, R., Strauss, S.H. and Busov, V.B. (2017) BIG LEAF is a regulator of organ size and adventitious root formation in poplar. *PLoS One*, **12**, e0180527.

Zhang, H., Harry, D.E., Ma, C., Yuceer, C., Hsu, C.-Y., Vikram, V., Shevchenko, O. *et al*. (2010) Precocious flowering in trees: the FLOWERING LOCUS T gene as a research and breeding tool in Populus. *J. Exp. Botany*, **61**, 2549–2560.