Highly efficient C-to-T and A-to-G base editing in a Populus hybrid

Gen Li1,1, Simon Sretenovic1,1†, Edward Eisenstein2,3, Gary Coleman1,2,* and Yiping Qi1,2,†

1Department of Plant Science and Landscape Architecture, University of Maryland, College Park, Maryland, USA
2Institute for Bioscience and Biotechnology Research, University of Maryland, Rockville, Maryland, USA
3Fischell Department of Bioengineering, University of Maryland, College Park, Maryland, USA

CRISPR-Cas-based genome editing technologies hold great potential for genetic research and bioengineering in trees. CRISPR-Cas9 was previously demonstrated to be a highly efficient genome editing system in poplar (Zhou et al., 2015). However, these applications only used the nuclease activity of Cas9 for targeted mutagenesis. By contrast, dead Cas9 (dCas9)-derived base editors can install specific base changes in a plant genome. Cytosine base editors (CBEs) can introduce C-to-T and occasionally C-to-G base changes (Komor et al., 2016; Nishida et al., 2016) and adenine base editors (ABEs) can generate A-to-G base changes (Gaudelli et al., 2017). CBEs can be applied for constructing premature stop codons, while both CBEs and ABEs can be used for making amino acid codon changes or altering RNA splicing sites or cis-regulatory elements in tailored applications. Despite their promising potential, base editing tools have not been fully established in trees.

To develop efficient base editors in trees, we worked on a Populus hybrid (Populus tremula × P. alba hybrid clone INRA 717-184). We first tried to establish an efficient C-to-T base editing system by comparing two promising CBEs, PmCDA1-BE3 and A3AY130F-BE3, which were in a base editor 3 (BE3) configuration by fusion of a cytidine deaminase and a uracil DNA glycosylase inhibitor (UGI) to the Cas9D10A nickase (Figure 1a). A PmCDA1 base editor in the BE3 configuration was previously demonstrated in rice (Tang et al., 2018). Recently, high-efficiency base editing in rice, wheat and potato was demonstrated with A3A-BE3 (Zong et al., 2018). Both PmCDA1-BE3 and A3A-BE3 showed much higher editing efficiencies than the widely used rAPOBEC1-BE3 in plants (Tang et al., 2018; Zong et al., 2018). Further, introduction of the Y130F mutation to A3A-BE3 appeared to be even more potent in making C-to-T conversions in human cells, especially at methylated target sites (Wang et al., 2018). Therefore, A3A/Y130F-BE3 is a promising CBE for testing in plants.

To compare PmCDA1-BE3 and A3AY130F-BE3, we targeted four sites in poplar with two sgRNAs editing 4-coumarate:CoA ligase 1 (4CL1) (4CL1-sgRNA1 and 4CL1-sgRNA2) and the other two sgRNA editing PII (PII-sgRNA1 and PII-sgRNA2), which encodes an evolutionarily conserved protein involved in coordinating carbon and nitrogen assimilation (Figure 1b). All four sgRNAs were designed to target both P. alba and P. tremula genomes and were intended to introduce premature stop codons. To effectively assess the CBEs, we constructed multiplexed T-DNA constructs where the two sgRNAs for editing the same gene were expressed under the ATU6 promoter and ATU3 promoter, respectively, and the Cas9D10A nickase-UGI fusion gene was expressed under the ATUbi10 promoter (Figure 1a). The four T-DNA vectors were constructed using our previously established multiplexed CRISPR assembly system (Lowder et al., 2015) and transformed into poplar petiole sections by Agrobacterium-mediated transformation (Leple et al., 1992). Between 19 and 22 T0 lines were randomly selected for each construct and then genotyped by Sanger sequencing. At 4CL1 A3AY130F-BE3 generated 50.0% and 95.5% editing frequencies with 4CL1-sgRNA1 and 4CL1-sgRNA2, respectively (Figure 1c). With PmCDA1-BE3, 26.3% and 78.9% editing frequencies were obtained for these two target sites, lower than those by A3A/Y130F-BE3 (Figure 1c). At both target sites, biallelic base editing lines were obtained with either A3A/Y130F-BE3 or PmCDA1-BE3 (Figure 1c). At PII, A3A/Y130F-BE3 and PmCDA1-BE3 generated 19.0% and 0% editing frequencies, respectively, with PII-sgRNA1 driven by the ATU6 promoter (Figure 1c). By contrast, PII-sgRNA2, driven by the ATU3 promoter, was of high efficiency as it resulted in 81.0% base editing frequency with A3AY130F-BE3 and 100% base editing frequency with PmCDA1-BE3 (Figure 1c). Altogether, we found both CBEs were highly efficient in generating targeted C-to-T base changes in poplar. Remarkably, we only identified insertion and deletion (indel) byproduct mutations at the 4CL1-sgRNA1 and PII-sgRNA2 target site (Figure 1c), suggesting high editing purity of both CBEs.

By combing the data from all four target sites in the T0 lines, it appeared that A3AY130F-BE3 can edit a broader window from C5 to C18 on the protospacer (Figure 1d). By contrast, PmCDA1-BE3’s editing window shifted slightly to the 5’ end of the protospacer, from C2 to C13 (Figure 1d). These observations were consistent with previous reports of editing windows for A3AY130F-BE3 in human cells (Wang et al., 2018) and for PmCDA1-BE3 in rice (Tang et al., 2018). We further conducted phenotypic investigation of four T0 lines edited by A3AY130F-BE3, with two being monoleptic mutants and the other two being biallelic mutants (Figure 1e). Since the biallelic mutants carried premature stop codons in both alleles of P. alba and P. tremula, we expected to see loss-of-function phenotype. Indeed,
Base editing in poplar
both biallelic mutants, not the monoallelic mutants, showed brown stems (Figure 1f), which suggested reduced lignin accrual and altered monolignol composition as previously reported for the loss of function of 4CL1 in poplar (Zhou et al., 2015).

Since its first demonstration in human cells (Gaudelli et al., 2017), A-to-G base editor ABEmax_V1 was further improved to ABEmax based on expression optimization and ancestral reconstruction (Koblan et al., 2018). To develop an ABE in poplar, we compared two ABEmax systems. These systems were based on human codon-optimized ecTadAwt-ecTadA* adenine deaminase, and maize or human codon-optimized Cas9D10A nickase, in ABEmax_V1 and ABEmax_V2, respectively (Figure 1a). Two targets sites at 4CL1, 4CL1-sgRNA5 and 4CL1-sgRNA6, were chosen for multiplexed A-to-G base editing (Figure 1a and b). Nineteen and 22 T0 lines were randomly selected for ABEmax_V1 and ABEmax_V2, respectively. Neither ABEs generated edited events with 4CL1-sgRNA5 which was driven by the ATU6 promoter (Figure 1c). However, with 4CL1-sgRNA6 driven by the ATU3 promoter, ABEmax_V1 and ABEmax_V2 generated 84.2% and 95.5% editing frequencies (Figure 1c), respectively, and many of the events are biallelic editing (Figure 1g and h). Notably, no indel byproducts were found among all T0 lines (Figure 1c), suggesting high-purity A-to-G conversions. Among seven adenines in the protospacer, A7 and A9 were both edited at high frequencies (Figure 1i). The data were in line with the general editing window from A4 to A10 for ABE systems (Gaudelli et al, 2017; Koblan et al., 2018).

In this study, we compared two CBEs and two ABEs in poplar for precise base editing. Based on the editing data from six independent multiplexed base editing constructs, the ATU3 promoter consistently yields much higher base editing frequencies (78.9%–100.0%) than the ATU6 promoter (0%–50.0%) (Figure 1a and c). Hence, with the ATU3 promoter for sgRNA expression, the CBEs and ABEs that we tested here should yield high-efficiency base editing and will have promising applications for precise genome editing in poplar and other trees.

Acknowledgements
This work was supported by the USDA BRAG programme (award nos. 2018-33522-28789 and 2020-33522-32274) and Emergency Citrus Disease Research and Extension Program (award no. 2020-70029-33161) to Y. Q; the USDA-NIFA grant (award no. 2019-67013-29197) to G.C. and E.E.; the McIntire-Stennis project (award no. MD-PSLA-20006) to G.C.; and the DOE BER programme (award no. DE-SC0017886) to E.E. S. S. is a Foundation for Food and Agriculture Research Fellow.

Conflicts of interests
The authors declare no competing financial interests.

Author contributions
Y.Q. and G.C. designed the study. Y.Q., G.C. and E.E. supervised the study. G.L. and S.S. performed the experiments and analysed the data. Y.Q., G.L., S.S., E. E. and G.C. wrote the paper.

References
Gaudelli, N.M., Komor, A.C., Rees, H.A., Packer, M.S., Badran, A.H., Bryson, D.I. and Liu, D.R. (2017) Programmable base editing of A to G in genomic DNA without DNA cleavage. Nature 551, 464–471.
Koblan, L.W., Doman, J.L., Wilson, C., Levy, J.M., Tay, T., Newby, G.A., Maianti, J.P. et al. (2018) Improving cytidine and adenine base editors by expression optimization and ancestral reconstruction. Nat. Biotechnol. 36, 843–846.
Komor, A.C., Kim, Y.B., Packer, M.S., Zuris, J.A. and Liu, D.R. (2016) Programmable editing of a target base in genomic DNA without double-stranded DNA cleavage. Nature 533, 420–424.
Leple, J.C., Basileiro, A.C., Michel, M.F., Delmotte, F. and Jouanin, L. (1992) Transgenic poplars: expression of chimeric genes using different vector constructs. Plant Cell Rep. 11, 137–141.
Lowder, L.G., Zhang, D., Baltes, N.J., Paul, J.W., Tang, X., Zheng, X., Voytas, D.F. et al. (2015) A CRISPR/Cas9 toolbox for multiplexed plant genome editing and transcriptional regulation. Plant Physiol. 169, 971–985.
Nishida, K., Araroe, T., Yachie, N., Banno, S., Kakimoto, M., Tabata, M., Mochizuki, M. et al. (2016) Targeted nucleotide editing using hybrid prokaryotic and vertebrate adaptive immune systems. Science 353, aaf8729.
Tang, X., Ren, Q., Yang, L., Bao, Y., Zhong, Z., He, Y., Liu, S. et al. (2018) Single transcript unit CRISPR 2.0 systems for robust Cas9 and Cas12a mediated plant genome editing. Plant Biotechnol. J. 17, 1431–1445.
Wang, X., Li, J., Wang, Y., Yang, B., Wei, J., Wu, J., Wang, R. et al. (2018) Efficient base editing in methylated regions with a human APOBEC3A-Cas9 fusion. Nat. Biotechnol. 36, 946–949.
Zhou, X., Jacobs, T.B., Xue, L.J., Harding, S.A. and Tsai, C.J. (2015) Exploiting SNPs for biallelic CRISPR mutations in the outcrossing woody perennial Populus reveals 4-coumarate-CoA ligase specificity and redundancy. New Phytol. 208, 298–301.
Zong, Y., Song, Q., Li, C., Jin, S., Zhang, D., Wang, Y., Qiu, J.L. et al. (2018) Efficient C-to-T base editing in plants using a fusion of nCas9 and human APOBEC3A. Nat. Biotechnol. 36, 950–953.

© 2021 The Authors. Plant Biotechnology Journal published by Society for Experimental Biology and The Association of Applied Biologists and John Wiley & Sons Ltd., 19, 1086–1088