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Supplementary information for A Dual-gRNA CRISPR/Cas9 System for Efficient Generation of Large Fragment Deletions in Poplar

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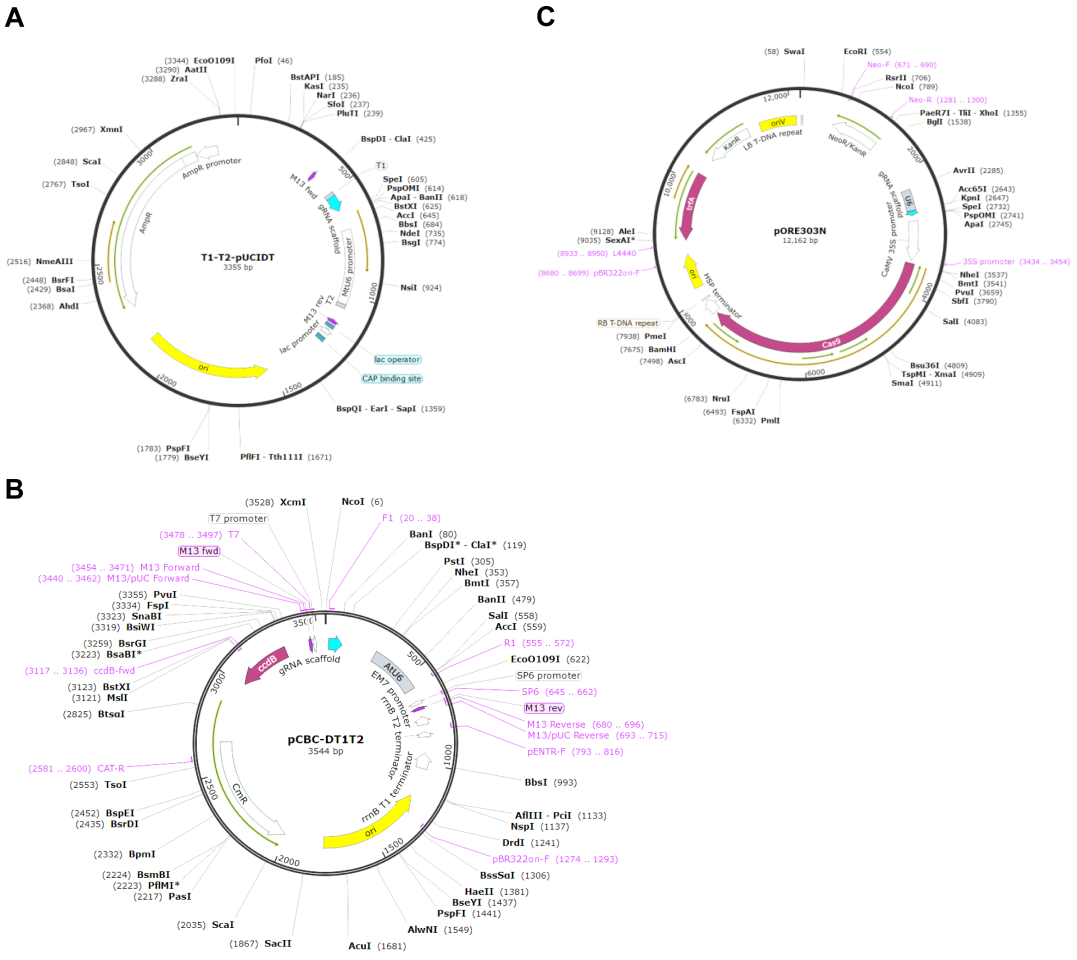

Figure S1. Detailed maps of the vectors used in this protocol. (A) Detailed plasmid map of the intermediate vector T1–T2–pUCIDT, showing the arrangement of the *Mt*U6 promoter, gRNA scaffold, and Target1/Target2

insertion sites used for dual-sgRNA cassette assembly. (B) Detailed plasmid map of the intermediate vector pCBC-DT1T2 (Xing et al., 2014), illustrating the *At*U6 promoter–driven sgRNA cassette. (C) Detailed plasmid map of the binary destination vector pORE303N (Bewg et al, 2022; Ortega et al, 2022), highlighting the T-DNA region, including the *Mt*U6 promoter, KpnI restriction site for gRNA cassette insertion, and the 2×35S-driven SpCas9 expression module. These detailed vector maps correspond to the simplified schemes shown in Figure 1.

Figure S2. Sequence confirmation of PCR fragment amplified from pCBC-DT1T2. The fragment contains the Target1 sequence, gRNA scaffold, *At*U6 promoter, and Target2 sequence, arranged for assembly of a dual-sgRNA cassette. Target1 and Target2 represent user-defined guide RNA target sequences that can be replaced with gene-specific targets of interest. Sequences highlighted in red denote the flanking homologous regions used for HiFi cloning. This sequence corresponds to the PCR product shown in Figure 1.

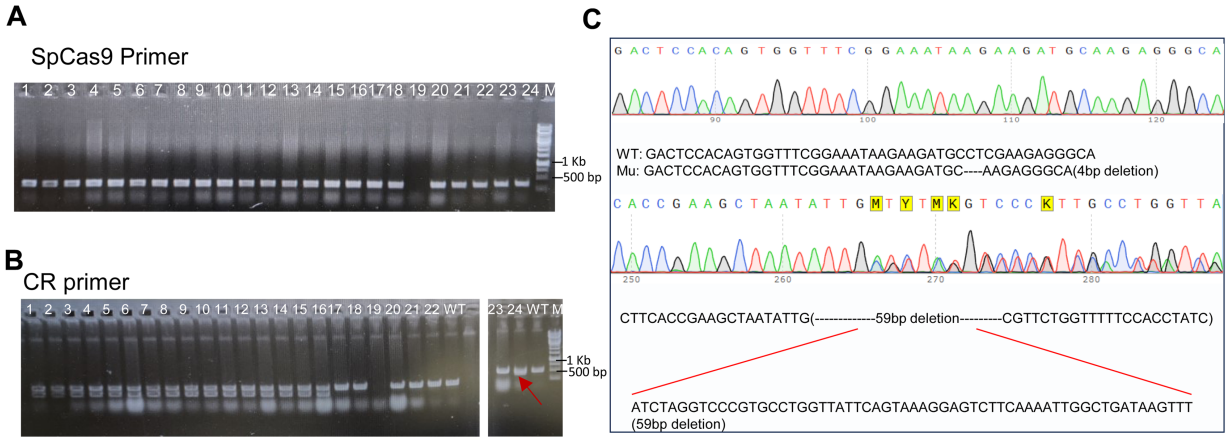

Figure S3. Genotyping of *PtFBX230-sg-pORE* transgenic lines generated using pCBC-DT1T2. (A) PCR amplification of the SpCas9 transgene using Cas9-specific primers. M, DNA size marker. Note that Line 19 did not yield a PCR product due to poor DNA quality. (B) PCR amplification of a DNA fragment spanning the two sgRNA target sites using CR primers (PtFBX230CR-iden-F and PtFBX230CR-iden-R) in independent transgenic lines and the wild-type (WT) control. Lines 1–18 display both wild-type and aberrant amplicons of altered sizes, consistent with chimeric editing events, whereas Line 24 exhibits a single shorter amplicon (arrow pointed), suggesting a homozygous or biallelic deletion event. Note that Line 19 did not yield a PCR product due to poor DNA quality and sample 23, 24 were run on a separate gel with an independent WT control. (C)

Sanger sequencing analysis of the PCR amplicon from Line 24, revealing a 4-bp deletion near Target1 and a 59-bp deletion near the Target2 region. The deleted nucleotide sequences of the targeted gene are shown in the lower panel. Letters K, Y, and W in the sequencing chromatograms represent degenerate bases derived from sequence polymorphisms between *P. tremula* and *P. alba*.