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Streamlined multiplex CRISPR editing of receptor-like kinases in *Populus* via cell suspension transformation

Dear Editor,

Receptor-like kinases (RLKs) constitute a large superfamily that regulates diverse biological processes in plants (De Smet et al., 2009). In the perennial tree *Populus*, nearly 200 RLKs are highly expressed in developing xylem, suggesting important roles in wood formation (Song et al., 2011; Zan et al., 2013; Xie et al., 2023). However, functional characterization of these genes has been hindered by their extensive redundancy and the practical difficulty of generating higher-order mutants at scale. Although CRISPR/Cas9 has been widely applied in herbaceous species (Lu et al., 2017; Meng et al., 2017; Chen et al., 2022), efficient multiplex editing across large gene families in *Populus* remains a major challenge. Conventional transformation methods using leaf discs or stem segments can yield precise edits (Fan et al., 2015; Bewg et al., 2022; Sulis et al., 2023), but they are not optimized for the rapid, high-throughput generation of multi-gene knockouts. To overcome this limitation, we developed a high-throughput suspension cell-based CRISPR/Cas9 platform that enables scalable integration of pooled constructs and rapid generation of multiplex mutant populations, addressing both functional redundancy and the constraints of traditional screening in *Populus*.

Traditional callus-derived suspensions tend to re-callus and aggregate during culture, which reduces transformation efficiency. To circumvent this, we established a novel and highly efficient system for inducing suspension cells directly from leaf tissue. This leaf-derived suspension system, developed for *Populus* (*Populus deltoides* × *P. euramericana* cv. "Nanlin895"), involves culturing leaf explants (1 cm × 0.2 cm) from 1-month-old seedlings in liquid Murashige and Skoog (MS) medium supplemented with 2, 4-dichlorophenoxyacetic acid (2,4-D) and sucrose. Under continuous light and constant shaking (120 rpm), rapidly dividing cells are released into the medium, leading to quick proliferation and the formation of a dense culture within 2–3 weeks (Figure 1A; Supplemental Figure 1). We then separated and subcultured the suspended cells for approximately 7 days (logarithmic growth phase) before *Agrobacterium*-mediated transformation (Supplemental Figure 2).

We tested whether transforming dispersed suspension cells with pooled constructs could generate diverse transgenic events in a single experiment. In an initial trial with 50 distinct constructs, up to 9 were integrated in a single transformation. We then evaluated smaller pools of 4, 6, 8, 10, and 12 constructs. Transforming suspension cells with these pooled *Agrobacterium* strains yielded 75–86 independent transgenic lines per pool, with construct integration frequencies ranging from 42% to 100% (Supplemental Figure 3). Although both four- and six-construct pools achieved a 100% integration rate, the four-construct pools produced the most balanced distribution of transgenic lines. These findings indicate that a pool size of four is optimal for population-scale functional genomics, as it maximizes the number of independent events per construct rather than stacking multiple vectors in single plants.

To enable high-throughput multiplex gene editing, we designed a CRISPR/Cas9 system with four distinct expression constructs. Each construct carried a unique sequential arrangement of four different U3/U6 promoters (U3b, U3d, U6-1, and U6-29), which drove four single guide RNAs (sgRNAs) targeting diverse genes (Supplemental Figure 4). This design broadens the targeting potential and simplifies genotype determination in pooled transformants. We then pooled the constructs and transformed the suspension cells during the logarithmic growth phase. The transformation achieved ~92% efficiency (57 transgene-positive lines out of 63 analyzed T_0 lines). Sequencing confirmed gene-editing efficiencies of 65%-72% across the targeted genes, regardless of the specific U3/U6 promoter arrangement utilized for the sgRNA cassettes (Supplemental Figure 5). These results demonstrate the feasibility of multiplex gene editing in suspension cells using pooled constructs.

To generate a collection of mutants targeting xylem-specific RLKs (Song et al., 2011; Zan et al., 2013; Xie et al., 2023), we designed 52 CRISPR/Cas9 constructs, each carrying four sgRNA cassettes targeting 2-8 RLK genes (Figure 1B; Supplemental Table 1). After verifying the target sequences in the *Populus* Nanlin895 background (Supplemental Table 2), we grouped the *Agrobacterium* strains carrying these 52 constructs into 13 pools (G1–G13, each containing four constructs) and transformed the suspension cells (Figure 1C). This generated 4093 independent T_0 transgenic lines (85% transgene-positive) targeting 182 distinct RLKs (Figure 1D and 1E). Each pool yielded 137–394 lines (Supplemental Figure 6). PCR analysis with primers specific to the U3b promoter (U3b-F) and vector backbone (SPR) confirmed integration of sgRNA cassettes, with over 40 independent lines obtained per construct (Figure 1D; Supplemental Figure 6). Among transformants, 93.4% carried one construct, and 6.6% carried two constructs (Figure 1D and 1E).

Sequencing of T_0 lines showed a 72.3% editing efficiency across target genes (Figure 1F). Of these, 37.3% harbored homozygous or biallelic mutations (Figure 1F; Supplemental Figure 7). The mutant population included single (25%), double (42.6%), triple (23.5%), quadruple (7.4%), and quintuple (1.5%) mutations (Figure 1G). Most were short 1–2 bp indels, representing 83.3% of edits (Figure 1H; Supplemental Figure 7). Analysis of

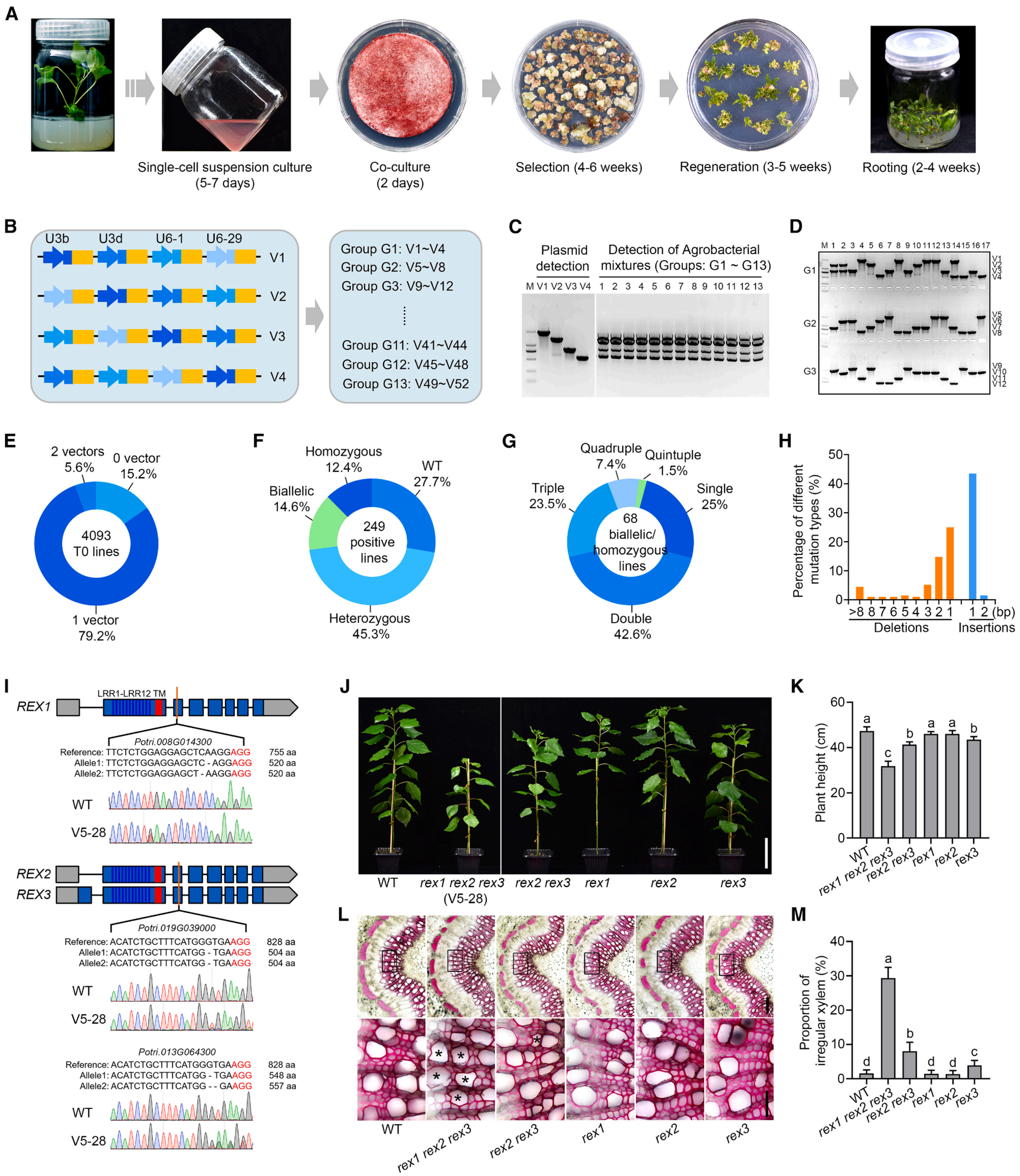

Figure 1. High-throughput generation of multiplex CRISPR/Cas9 receptor-like kinase mutants via cell suspension transformation in *Populus*.

(A) Workflow illustrating establishment of single-cell suspension cultures from *Populus* leaf explants, followed by *Agrobacterium*-mediated transformation and plant regeneration.

(B) Strategy for pooled transformation using 52 multiplex CRISPR/Cas9 constructs (V1–V52). Each construct contains four sgRNA expression cassettes targeting RLK genes. Constructs were organized into 13 pools (G1–G13), each containing four vectors. Representative designs (V1–V4) show unique promoter arrangements (U3b, U3d, U6-1, U6-29).

(C) PCR validation confirming plasmid integrity (V1–V4) and presence in pooled *Agrobacterium* batches (G1–G13). M, DNA marker.

(legend continued on next page)

predicted off-target sites revealed no detectable mutations, confirming the high specificity of our CRISPR/Cas9 editing system (Supplemental Table 3).

The RLK mutant set, enriched for single, double, and triple mutants, enabled functional analysis. For example, we identified single, double, and triple mutants of three paralogous RLKs: *Potri.008G014300*, *Potri.019G039000*, and *Potri.013G064300*, designated *RLK Expressed in Xylem 1*, *2*, and *3* (*REX1*, *REX2*, and *REX3*) (Figure 1I; Supplemental Figure 7). Single *rex1* and *rex2* mutants showed no phenotype, whereas the *rex3* single and *rex2 rex3* double mutants exhibited reduced growth. The *rex1 rex2 rex3* triple mutant (lines V5–28) displayed dwarfism and pale-green leaves (Figure 1J and 1K). Quantitative chlorophyll assays confirmed significant decreases in chlorophyll a and b relative to wild type and other genotypes (Supplemental Figure 8). Stem cross-sections showed thinner fiber walls and irregular xylem vessels in the triple mutant (Figure 1L and 1M; Supplemental Figure 9), indicating compromised secondary cell wall formation. These findings demonstrate functional redundancy among *REX1*, *REX2*, and *REX3* in xylem development.

While previous multiplex CRISPR strategies in *Populus* have successfully enabled pathway engineering and editing of conserved homologous genes (Bewg et al., 2022; Sulis et al., 2023), our platform offers a complementary approach designed for scale. By combining pooled-construct libraries with an efficient leaf-derived cell suspension system, we achieved rapid generation of large, low-mosaic mutant populations spanning entire gene superfamilies. This strategy is particularly suited to discovery-driven functional genomics, where the key challenge is producing large numbers of independent mutant lines across broad target sets to dissect complex and redundant gene functions in woody species.

In summary, we developed a suspension cell-based CRISPR/Cas9 platform that supports scalable and balanced multiplex editing of RLKs in *Populus*. The system enabled the rapid creation of large, diverse mutant populations, overcoming long-standing barriers to studying functional redundancy and gene families in woody plants. Beyond advancing basic research, this platform also provides a path to accelerate molecular breeding in woody perennials.

DATA AVAILABILITY

The pABE8e construct and RLK mutants generated in this study are available from the corresponding author upon request.

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The authors declare no conflicts of interest.

AUTHOR CONTRIBUTIONS
J.G., Junhui Shen, Jiayan Sun, Y.Z., Laifu Luo, X.L., Z.L., Z.G., S.C., Y.Z., and J.L. conducted the experiments. J.G. and Laigeng Li designed the experiments and wrote the paper.

SUPPLEMENTAL INFORMATION
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(D) PCR analysis verifying sgRNA cassette integration in representative T_0 lines from groups G1–G3.
(E) Distribution of the number of integrated constructs per T_0 line based on PCR screening of 4093 independent transgenic lines. Percentages indicate lines with 0, 1, or 2 integrated vectors.
(F) Gene-editing outcomes based on sequencing of 249 randomly selected T_0 lines. WT, wild type.
(G) Frequencies of single and multiple gene edits in the 68 confirmed biallelic/homozygous mutant lines identified in **(F)**.
(H) Distribution of insertion and deletion mutations observed at target sites in the edited lines.
(I) Genotyping of the *rex1 rex2 rex3* triple mutant line (V5–28). Schematics show gene structures along with sgRNA target sites. Sanger sequencing chromatograms confirm frameshift mutations leading to premature stop codons. Dashes indicate deleted bases; PAM sequences are in red. Numbers indicate amino acid length of the corresponding wild-type and truncated REX proteins.
(J) Phenotypes of 2-month-old WT and representative T_0 single (*rex1*, *rex2*, *rex3*), double (*rex2 rex3*), and triple (*rex1 rex2 rex3*) mutants grown in a phytotron. Bar, 10 cm.
(K) Plant height of WT and *rex* mutant lines. Data are means ± SD, n = 8 plants.
(L) Phloroglucinol-HCl staining of transverse sections from the 11th internode of 2-month-old plants. Asterisks mark misshapen xylem vessels in the *rex1 rex2 rex3* triple mutant. Bar, 200 μm (upper panel), 50 μm (lower panel).
(M) Proportion of irregular xylem in WT and *rex* mutants, calculated as misshapen vessels relative to total vessels. Data are means ± SD, n = 8 plants. Different lowercase letters in **(K)** and **(M)** indicate significant differences at $p < 0.01$ (ANOVA).

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