Brief Communication

Editing gene families by CRISPR/Cas9: accelerating the isolation of multiple transgene-free null mutant combinations with much reduced labor-intensive analysis

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Genetically elucidating functions of gene families often requires simultaneous disruption of several family members to overcome redundancy. CRISPR/Cas9-based gene editing technology can simultaneously knockout multiple target genes (Armario Najera et al., 2019; Li et al., 2021; Ma et al., 2015), but it is laborious to isolate multiple null combinations lacking transgenes (Miao et al., 2018; Zhao et al., 2018). Removal of transgenes is necessary because the CRISPR components can generate new mutations and off-target events in next generations (Zhang et al., 2014, 2018). Genetic segregating out the transgenes in a multiplexing experiment is very time consuming and labor intensive, because the desired lines rarely only have just one T-DNA insertion (Luo et al., 2021).

We developed an effective approach to generate various transgene-free knockout mutant combinations with greatly reduced labor and time involved. For comparison, we constructed a traditional multiplex CRISPR vector MCK to target four genes (Figure 1a). In theory, 75% of the T1 plants contained the transgenes (Figure 1b). The MCK vector was effective in editing target genes and many T1 plants had mutations in all target genes including two T1 plants that were null at all four target genes. However, none of the transgene-free T1 plants were truly null at all target genes. We do not consider that plants with a single base pair (bp) substitution or deletions of 3× bp are null (Figure 1c).

We previously developed the TKC (Transgene Killer CRISPR) technology that relies on temporal expression of two suicide cassettes (Figure 1d) that kill all transgene-containing embryos and sperms (He et al., 2018). We targeted several genes from the NAKED PINS IN YUC MUTANTS (NPY) (Cheng et al., 2007) family in rice using TKC (Figure 1e). We focused on conducting genetic analyses on progenies from T0 plants with the seed setting rate <50%, which is a good indication of effectiveness of TKC (Figure 1f). The 13 T1 plants produced from self-pollination of TKC-M1 T0 plants did not contain the transgenes based on PCR-based genotyping (Figure 1g). The Sanger sequencing results of T1 plants revealed several interesting patterns: (i) Simultaneous disruption of the four target genes was efficiently achieved. Among the 13 T1 plants analyzed, eight of them were quadruple mutants (Figure 1g), (ii) The gene-editing efficiency differed significantly among the four target genes (Figure 1g), (iii) GENE1, GENE2, and GENE3 were not null in nine T1 plants of the TKC-M1 because they contained deletions of 3 or 9 bp. The desired null mutant combinations in all targets were actually in minority (2/13, 15.4%; Figure 1g). If MCK is used, the chance to identify transgene-free null mutant would decrease to below 3.85% (25% × 15.4%).

We constructed a second multiplex TKC vector TKC-M2 to target GENE1, GENE5, GENE6, and GENE7 to further test the effectiveness of our approach. The 16 T1 plants produced from eight T0 TKC-M2 plants (TKC-M2#1 to TKC-M2#8) were all transgene-free (Figure 1h). The target genes had been edited in all but one T1 plants. We obtained three transgene-free quadruple null mutant combinations (3/16, 19%). Surprisingly, one of the quadruple mutants (TKC-M2#6) was homozygous in GENE7 with an insertion of a gRNA backbone fragment (Figure 1h).

To further verify the effectiveness of our approach and to generate useful mutant combinations of NPY-like genes, which are involved in auxin-mediated organogenesis, we targeted GENE8 to GENE12 that are very closely related in a phylogenetic tree (Figure 1e). The TKC-M3 vector targeted five genes. The first gRNA targeted both GENE8 and GENE9 (Figure 1i). None of the 71 T1 plants from TKC-M3 contained transgenes (Figure 1i). The sequencing results in randomly chosen T1 plants are shown in Figure 1i. GENE8 and GENE9 were not edited as efficiently as GENE10 and GENE12. We obtained various transgene-free null mutant combinations (Figure 1i), which provide valuable materials for further studying the NPY-like genes. We were disappointed that simultaneous disruption of the five target genes was not successful. One potential explanation for the lack of homozygous quintuple mutants is that the mutants are lethal. We germinated T1 seeds of TKC-M3#2 and TKC-M3#5 and found that the germination rates of the seeds were normal (Figure 1i). Furthermore, no homozygous gene9 mutants were identified (Figure 1i), indicating that the homozygous mutations of GENE9 likely caused lethality, which was also consistent with...
the observation that the segregation ratio between plants with wild-type *GENE9* and heterozygote *gene9* was about 1:1 (Figure 1j).

In conclusion, we demonstrated an effective approach for isolating various transgene-free null mutant combinations of gene family members (Figure 1k). Our approach greatly decreases the time and labor needed and providing useful tools for studying gene families and genetic interactions.

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Conflict of interest

The authors declare no conflicts of interest.

Author contributions

N.Y., L.Y., Y.H., and Y.Z. conceived the idea and wrote the first draft of the manuscript. N.Y., L.Y., Z.Z., Y.Z., Y.T., T.Z., R.L., X.G., and Y.H. conducted the experiments. H.Z., M.X., M.Z., and J.W. contributed to manuscript revision.

Figure 1 Reduced labor required for generating various transgene-free null mutant combinations. (a) A schematic representation of the key components of a traditional CRISPR MCK. (b) Identification of transgene-free T1 plants by PCR. (c) Sequencing results of transgene-free MCK T1 mutants. ‘S3’ refers to three base pairs substitution. (d) Schematic representations of the key components of a TKC. (e) Phylogenetic analysis of NPY1 homologs from rice and Arabidopsis. (f) Seed setting rate of T0 plants of TKC. (g–i) Transgene-free multiple mutants produced by TKC-M1, TKC-M2, and TKC-M3, respectively. Primer pairs ‘RG’ and ‘TG’ are used to check the quality of rice genome DNA and to identify the presence of T-DNA, respectively. ‘-’ refers to a deletion of one base pair. A letter in magenta and superscript refers to an insertion of a base. ‘S1’ means one base pair substitution. The plants with null mutation *Cas9* and traditional CRISPR. Magenta indicates a transgenic element with heterozygous, homozygous, and biallelic plants, respectively. (k) A simulated diagram of the inheritance of multiple loci mutation in rice generated by TKC and traditional CRISPR. Magenta indicates a transgenic element with Cas9. Transgene-free plants were marked with cyan. Other colors represent the chromosomes where the genes targeted by gRNAs are located, respectively.

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