

RESEARCH ARTICLE OPEN ACCESS

TKC-MC: An Effective Strategy for Generating Heritable Heterozygous Mutations in Essential Genes in Rice

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Received: 2 September 2025 | **Revised:** 27 October 2025 | **Accepted:** 13 November 2025

Keywords: CRISPR/Cas9 | mismatch-spacer | rice | TKC | transmission essential gene

ABSTRACT

The CRISPR/Cas9 gene-editing technology has been widely used in defining gene functions and crop improvement. However, some genes are essential for plant growth and development. Loss-of-function homozygous mutations in essential genes lead to plant death or sterility. Mutations in essential genes need to be maintained and propagated in heterozygous plants. CRISPR/Cas9 technology is highly efficient in generating homozygous or bi-allelic mutations at T0 generation in rice, making it difficult to generate useful genetic materials for essential genes using traditional gene editing technology. In this study, we designed Transgene-Killer CRISPR (TKC)-mediated mismatch-spacer targeting (TKC-M) to efficiently generate heritable heterozygous mutations in essential genes in rice. Leveraging our earlier transgenic offspring self-elimination TKC platform, TKC-M relied on timely self-elimination of Cas9 and engineered gRNA-target mismatches to enrich heritable heterozygous or mosaic incomplete-edited T0 mutants and heterozygous progeny. We found that the sensitivity of targets to spacer mismatch(es) varies. A single-base mismatch at gRNA positions 11 or 17 yielded abundant heritable heterozygotes in sensitive targets. For insensitive targets, dual mismatches at positions 8 and 15 maximised heritable heterozygotes. Co-transformation of rice with TKC vectors carrying gRNA without mismatches (G1), gRNA with a mismatch at position 11 (M11) and M8 + M15 spacers, termed TKC-M Cocktail (TKC-MC) significantly increased the incomplete-edited mutant ratio compared with using G1 alone. This work establishes a technical foundation for generating mutant libraries that cover every single gene in a plant genome and for in-depth research on essential genes.

1 | Introduction

Loss-of-function mutations are required to define gene functions and recent advancements in CRISPR/Cas9 gene editing technologies have made it easier to generate knockout mutants in many plants. However, some genes are so crucial for normal plant growth and development, named transmission essential genes (TEGs). Knockout mutations in TEGs often lead

to sterility or lethality. For example, mutations in TEGs can cause failure to germinate, inability to differentiate into seedlings through tissue culture, premature death during growth (Xu et al. 2015; Hu, Miller, et al. 2018; Hu, Tian, et al. 2018; Wang et al. 2024), inability to survive because of albino seedlings (Andrieu et al. 2012; Lin et al. 2015; Xu et al. 2024) and female sterility (He et al. 2019; Lu et al. 2020). In rice, CRISPR/Cas9 is very efficient at generating homozygous or

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bi-allelic mutations at the T0 generation, making it difficult to use gene-editing technologies to create heritable mutations in TEGs. A lack of stable mutant materials hinders in-depth analysis of gene functions. Among the 30 000–50 000 genes in the rice genome, only 4500 rice genes have been cloned and functionally studied (Yao et al. 2018; Huang et al. 2022). Loss-of-function mutations in some genes do not lead to a phenotype due to genetic redundancy (Li et al. 2011; Zhang et al. 2018). Some genes are essential and homozygous mutations cause lethality or sterility. Consequently, very few female-sterile mutants have been isolated. We previously generated a stigma-less rice mutant through reverse genetics (He et al. 2019). Heterozygous knockouts, however, grow normally (Hu, Miller, et al. 2018; Hu, Tian, et al. 2018; He et al. 2019), providing stable material for in-depth and rigorous gene-function studies.

The Cas9/gRNA complex cleaves genomic DNA, and the error-prone non-homologous end-joining (NHEJ) repair often leads to a small deletion/insertion that causes frameshift and inactivates the target gene (Makarova et al. 2011; Jinek et al. 2012; Liu et al. 2017; Zhu 2022). Continuous presence of the Cas9/gRNA complex often leads to homozygous or bi-allelic mutations in rice and other diploid plants (Zhou et al. 2014; Ma et al. 2015; He et al. 2017; Wang et al. 2017), making it difficult to generate viable plants with heritable mutations in TEGs. One strategy to generate mutations in TEGs is to target 3'-terminal (Zhang et al. 2024) or to generate in-frame indels of TEGs (Minkenberg et al. 2017; Lee et al. 2020). However, the mutations obtained in these ways are partial loss-of-function and require extensive screening. Therefore, developing a broadly applicable method to easily and efficiently create heritable full loss-of-function mutants will be of great value. Because standard CRISPR/Cas9 typically drives complete editing (Zhou et al. 2014; Ma et al. 2015), lowering Cas9 efficiency is a plausible route to preserving heterozygous mutants. Editing efficiency is governed by (i) promoter strength (Gao and Zhao 2014; He et al. 2017; Gao et al. 2019; Xie and Yang 2019), (ii) rational design and modification of the gRNA molecule (Fu et al. 2014; Ren et al. 2014; Doench et al. 2016; Liu et al. 2016; Sun et al. 2025); (iii) the cutting ability of Cas protein variants (Kleinstiver et al. 2016; Hu, Miller, et al. 2018; Hu, Tian, et al. 2018; Liu et al. 2020; Walton et al. 2020). Perfect spacer-target pairing is critical to the function of the CRISPR/Cas9 system. Mismatches between the spacer and target lower Cas9/gRNA affinity and alter Cas9 conformation (Lin et al. 2014; Zheng et al. 2017; Bravo et al. 2022; Pacesa et al. 2022). Single mismatches are tolerated in the protospacer adjacent motif (PAM)-distal region (Jinek et al. 2012), whereas two or more mismatches near the PAM severely impair cleavage (Fu et al. 2013; Hsu et al. 2013; Tsai et al. 2014). Therefore, introducing spacer mismatch(es) offers a simple, tunable way to reduce Cas9 activity and to increase the proportion of heterozygous heritable knockouts.

In conventional CRISPR/Cas9 experiments, the persistent presence of Cas9/gRNA at T0 generation and T1 generation leads to further editing of the residual wild-type (WT) allele, converting heterozygotes to homozygous or biallelic states, preventing null mutations in TEGs from transmitting to the T1 or subsequent generations. We previously developed Transgene Killer CRISPR (TKC) technology (He et al. 2018; He and Zhao 2020), which

enables the rapid elimination of transgenic elements from edited rice lines within a single generation. Self-elimination of Cas9 by TKC allows the recovery of T1 plants with desired mutations and no further editing. This technology allows rapid stacking of mutations in redundant gene families (Yang et al. 2022). Moreover, TKC coupled with the RUBY reporter (He, Zhang, et al. 2020) for visual tracking of transgene escape, further streamlines workflows (Zhu et al. 2025). We believe that TKC technology can help generate stable and heritable heterozygous mutations in TEGs.

In this study, we used spacer-engineered gRNAs to elicit incomplete editing of TEGs in T0 plants and coupled this approach with the TKC technology to secure heritable heterozygous progeny. Systematic profiling across loci revealed position-dependent effects of single-base mismatch: at sensitive targets, a mismatch at spacer positions 11 or 17 enriched incomplete-edited individuals. Whereas mismatches at spacer positions 8 and 15 proved optimal for less-sensitive targets, this combination consistently yielded a high proportion of incomplete edits. However, triple mismatches failed to edit target genes. Combining TKC and mismatches in gRNAs prevents target genes from further editing and locks the desired heterozygous state into subsequent generations. Because the effect of a single mismatch on a new target is unpredictable, we devised TKC-M Cocktail (TKC-MC): mixtures of *Agrobacterium* carrying TKC vectors and gRNA without mismatch (G1), a single-mismatch at position 11 (M11) and double mismatches at positions 8 and 15 (M8 + M15) were co-delivered to rice calli. Co-transformants exhibited a markedly higher frequency of incomplete-edited plants than controls transformed with the perfectly matched spacer alone, and the retained incomplete edits were almost exclusively driven by M11 or M8 + M15 gRNAs. TKC-MC therefore offers a robust, scalable strategy for obtaining stable and heritable mutants of TEGs.

2 | Results

2.1 | Single-Base Mismatches Between Spacer and Target Yield High Frequencies of Incomplete-Edited Mutants

To test the feasibility of our method, we selected three typical TEGs: *OsPDS*, when mutated, leads to albino and seedling lethality (Fang et al. 2008; Banakar et al. 2020), *OsNDUFA9* that leads to embryonic lethality when mutated (Hu, Miller, et al. 2018; Hu, Tian, et al. 2018) and *OsPID*, which causes female sterility when compromised (He et al. 2019; Xu et al. 2019; Wu et al. 2020). We first tested a series of single-base mismatches in the spacer sequence of the *OsPDS*-TS1 target site (Figure 1A). For T0 plants, we first determined whether they are transgenic and analyzed the mutations in the target genes (Figure 1B,C, Figure S1). We found that some vectors with mutated spacers yielded higher ratios of incomplete editing. For instance, *OsPDS*-TS1-M14 showed the highest proportion (56.67%), followed by M15 (28.57%) and M19 (27.27%) (Figure 1C, Table S1). Additionally, we found that single-base mismatches occurring in the first 10 bases of the spacer had a smaller impact on gene-editing efficiency (Figure 1C). Consistent with previous studies, the 10 nucleotides (nts) near the PAM in the spacer are the seed sequences

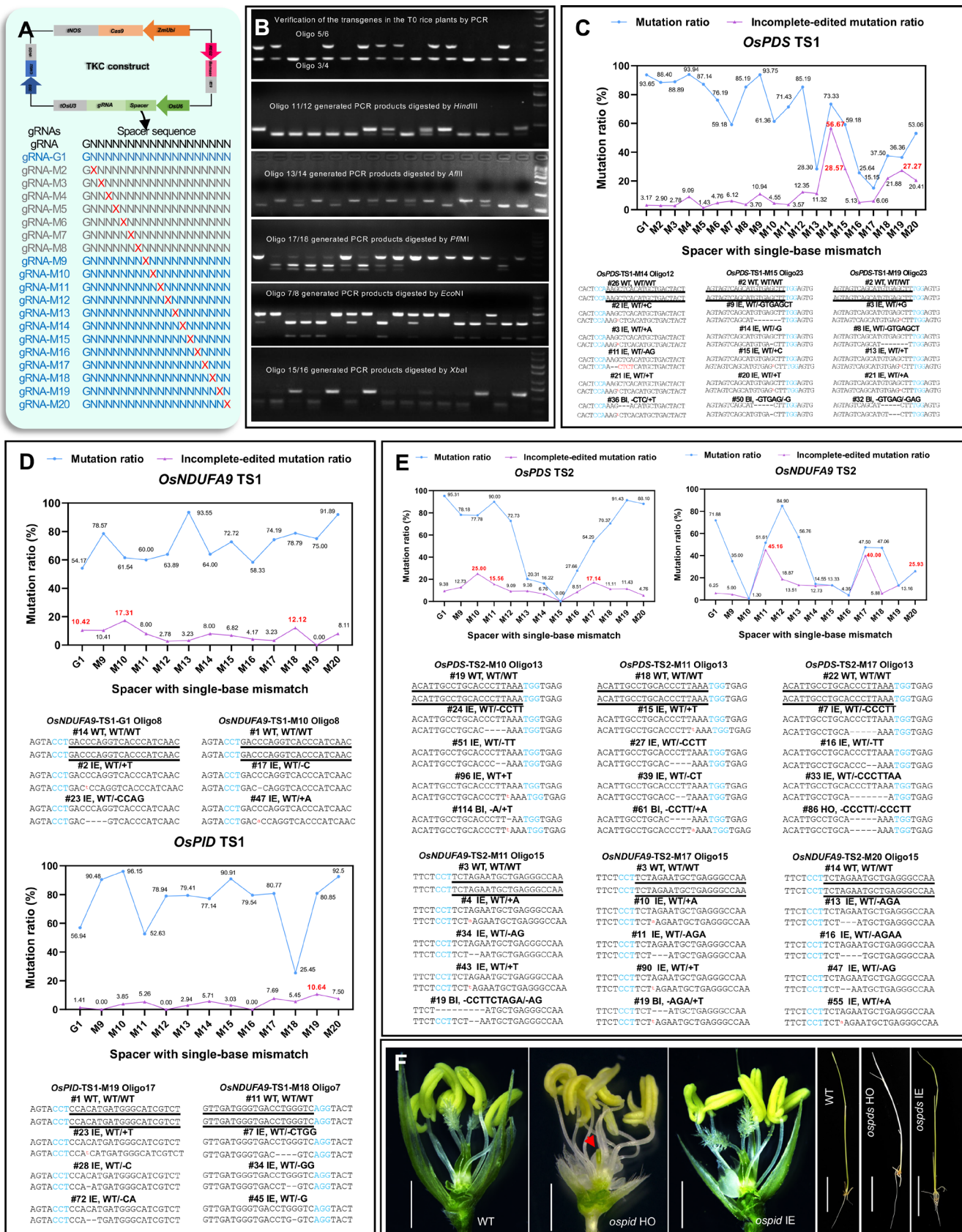


FIGURE 1 | Target editing TEGs by *TKC* constructs containing spacers with single-base mismatch. (A) A schematic representation of editing by Cas9/gRNA complex with single-base mismatch in the spacer. All constructs were based on the *TKC* (Transgene Killer CRISPR) plasmid. In the gene-editing components, the rice codon-optimised *Cas9* was placed under the control of the maize *ubiquitin* promoter. The gRNA was driven by rice snRNA promoter *OsU6c*. The suicide unit consists of two sub-units: The *CMS2* under the control of *CaMV 35S* promoter eliminates transgene-containing pollen grains. The *BARNASE* gene controlled by an early embryogenesis-specific promoter *REG2* causes the death of transgene-harboring embryos. The gRNAs with single-base mismatches are showed. The mismatched bases are highlighted with 'X' in red, which refers to 'A', 'T', 'C' or 'G'. (B) Transgenic positive identification and genotyping of T0 plants. 'Oligo 3/4' refers to the primer pair for checking the quality of rice genomic DNA. Primer pair Oligo 5/6 detects the T-DNA cassette. 'Oligo 11/12' genotypes T0 plants edited with OsPDS-TS1. 'Oligo 13/14' indicates the primer pair for genotyping T0 plants carrying OsPDS-TS2. 'Oligo 17/18' refers to the primer pair used for genotyping T0 plants targeting *OsPID* by OsPID-TS1. 'Oligo 7/8' represents the primer pair used for genotyping T0 plants containing OsNDUFA9-TS1 targeting to *OsNDUFA9*. 'Oligo 15/16' means the primer pair used for genotyping T0 plants targeting *OsNDUFA9* via OsNDUFA9-TS2. (C–E) The mutation ratios and representative mutation forms of the transgenic plants at the targets of OsPDS-TS1 (C), OsNDUFA9-TS1 and OsPID-TS1 (D) and OsPDS-TS2 and OsNDUFA9-TS2 (E). Blue lines represent mutation ratio = (number of homozygous mutants and incomplete-edited mutants)/number of transgene plants. Magenta lines mean incomplete-edited mutation ratio = incomplete-edited mutants/number of transgene plants. Relatively high incomplete-edited mutation ratios are highlighted in red. The representative sequencing results are shown below the mutation ratio plot. Underlined sequences correspond to the Spacer sequences. The PAM sites are marked in light blue. WT refers to wild-type Zhonghua 11 plants. 'IE' means incomplete-edited mutants. 'HO' represents homozygous mutants. 'BI' indicates biallelic homozygous mutants. '-' represents deletion of base pair(s). 'a', 't', 'c' and 'g' in red and superscript refer to an insertion of an 'A', 'T', 'C' and 'G', respectively. (F) The phenotypes of *ospid* and *ospds*. Both the *ospid* IE and *ospds* IE mutants show normal phenotypes as wild-type. As expected, the *ospid* HO lacks stigmas (red arrowhead). Scale bar, 0.2 cm. The *ospds* HO mutant displays an albino phenotype. Scale bar, 2 cm. 'WT' represents wild-type. 'IE' refers to incomplete-edited mutants. 'HO' means homozygous mutants.

within the seed sequence region are expected to produce a high ratio of incomplete-edited mutants.

Subsequently, we further tested four other target sites: OsNDUFA9-TS1, OsPID-TS1, OsPDS-TS2 and OsNDUFA9-TS2. A series of single-base mismatches were mainly set in the PAM-proximal 12 nt region containing the seed sequence (M9–M20). Our data showed that for the OsNDUFA9-TS1 and OsPID-TS1 target sites, after a series of single-base mismatches between the spacer and the target site, the overall mutation ratios remained high (blue lines in Figure 1D). We named these targets with low sensitivity to mismatch as targets with less sensitivity to mismatch (TLSM). The incomplete-edited mutation ratios for these TLSM targets were very low (magenta lines in Figure 1D). The highest incomplete-edited mutation ratio for OsNDUFA9-TS1 was only 17.31% (Figure 1D, Table S1). The incomplete-edited mutation ratio for OsPID-TS1 was even lower, with a maximum of only 10.64% (Figure 1D, Table S1). For these TLSM targets, single-base mismatches within the seed sequence region of the spacer did not effectively increase the ratio of incomplete-edited mutants, so other solutions were needed to increase the proportion of incomplete-edited mutants. However, for the OsPDS-TS2 and OsNDUFA9-TS2 target sites, single-base mismatches had a significant impact on gene-editing efficiency (blue lines in Figure 1E), named targets with high sensitivity to mismatch (THSM). Some single-base mismatches could produce a higher ratio of incomplete-edited mutants (magenta lines in Figure 1E). For example, for the OsPDS-TS2 target site, the three spacers with the highest incomplete-edited mutation ratios were M10, M11 and M17, with incomplete-edited mutation ratios of 25.0%, 15.56% and 17.14%, respectively, which are higher than the 9.38% from gRNA without mismatch (Figure 1E, Table S1). For the OsNDUFA9-TS2 target site, the M11 spacer exhibited the highest incomplete-edited mutation ratio (45.16%), followed by M17 (40.00%) and M20 (25.93%), all significantly exceeding that of the control G1 (6.25%) (Figure 1E, Table S1). From the above data, we concluded that for THSM targets such as OsPDS-TS1, OsPDS-TS2 and OsNDUFA9-TS2, single-base mismatches

within the seed sequence region of the spacer could adequately increase the proportion of incomplete-edited mutants. The most frequently occurring mismatch types were M11 and M17. We selected M11 for further application in our subsequent research.

2.2 | Dual-Base Mismatches Between Spacer and Target Enhance Incomplete-Editing Frequency in TLSM Targets

Single-base mismatches within the seed sequence produced a low proportion of incomplete-edited mutations in TLSM targets such as OsNDUFA9-TS1 and OsPID-TS1 (Figure 1D). To address this deficiency, we attempted to introduce double or triple-base mismatches within the spacer region. Additionally, we also tested the THSM target OsPDS-TS1 to compare the effects of multiple-base mismatch with single-base mismatches. We first introduced triple-base mismatches in the OsNDUFA9-TS1 and OsPDS-TS1 targets (Figure 2A). We performed transgenic positive identification and target gene mutation identification for the T0 plants (Figure 2B, Figure S2). The results showed that all tested triple-base mismatches between the spacer and the target site led to a complete failure in editing the target sites (Figure 2C). Therefore, we attempted to design double-base mismatches between the spacer and the target site for OsNDUFA9-TS1, OsPDS-TS1 and OsPID-TS1 targets (Figure 2A). Previous studies have identified a 'core sequence' of four positions (14–17) within the spacer where mismatch has the greatest impact on editing efficiency (Zheng et al. 2017). In our study, we found that in THSM targets, single-base mismatches that reduced editing efficiency to below 20% were also mainly located within this core sequence. For example, in OsPDS-TS1, the mismatch was at position 17, in OsPDS-TS2, the mismatches were at positions 14, 15, and in OsNDUFA9-TS2, the mismatches were at positions 10, 14, 15, 16 and 19 (Figure 1C,E). Therefore, to reduce the amount of testing, we chose one of the frequently occurring positions, either 14 or 15, and focused on position 15, and then combined it with another position to

FIGURE 2 | Incomplete-editing efficiencies improvement by spacers with multiple-base mismatches. (A) A schematic representation of editing by Cas9/gRNA complex with multiple-base mismatches in the spacer. Symbols as in Figure 1A. (B) The representative sequencing results of T0 transgenic plants edited by *TKC* construct with dual mismatch spacer M8 + M15. The PAM sites are marked in light blue. ‘WT’ represents wild-type Zhonghua 11 plants. ‘IE’ refers to incomplete-edited mutants. ‘BI’ indicates biallelic homozygous mutants. ‘-’ refers to deletion of one base pair. ‘a’, ‘t’, ‘c’ and ‘g’ in red and superscript indicate an insertion of an ‘A’, ‘T’, ‘C’ and ‘G’, respectively. (C) Mutation statistics of the transgenic plants at the targets of OsPDS-TS1, OsNDUFA9-TS1 and OsPID-TS1. Blue lines indicate mutation ratio = (number of homozygous mutants and incomplete-edited mutants)/number of transgene plants. Magenta lines refer as to incomplete-edited mutation ratio = incomplete-edited mutants/number of transgene plants. Relatively high incomplete-edited mutation ratios are highlighted in red.

create double-base mismatches. For the OsPDS-TS1 target site, the double-base mismatch spacer M8 + M15 yielded the highest proportion of incomplete-edited mutants (32.76%), surpassing the single-base mismatch M15 (28.57%) but still lower than that of M14 (56.67%) (Figures 1C and 2C, Tables S1 and S2). This indicates that in THSM targets, single-base mismatch variant spacers are sufficient to achieve a high proportion of incomplete-edited mutants. For the OsNDUFA9-TS1 target site, double-base mismatches variant spacers such as M7 + M15, M8 + M15, M9 + M15 and M10 + M15 produced incomplete-edited mutant ratios all exceeding 30%, with the M8 + M15 variant spacer producing the highest ratio of incomplete-edited mutants at 61.11% (Figure 2C, Table S2). Additionally, for the OsNDUFA9-TS1 and OsPDS-TS1 targets, when the designed double-base mismatches were located at the core sequence and the PAM-proximal region, such as the double-base mismatches variant spacers M14 + M17 and M14 + M19, they almost failed to edit the target sequence (Figure 2C, Table S2).

We subsequently designed double-base mismatches with position 15 plus another position for the OsPID-TS1 target site. We found that in the OsPID-TS1 target site, double-base mismatches

containing a base mismatch at position 15 could still efficiently edit the target gene (blue lines in Figure 2C), indicating that this target site has a high tolerance for spacer mismatches and can maintain high editing efficiency even with two-base mismatches in the PAM-proximal region. However, the incomplete-edited mutant ratios produced by the double-base mismatches variant spacers M8 + M15 and M10 + M15 exceeded 20%, reaching 35.85% and 40.00%, respectively (Figure 2C, Table S2), which could not be achieved with single-base mismatches. Therefore, we concluded that for TLSM targets, single-base mismatches between the spacer and the target site are unlikely to produce a high proportion of incomplete-edited mutants. Designing double-base mismatches with one-mismatch position in the ‘core sequence’ region can effectively increase the ratio of incomplete-edited mutants, with the M8 + M15 double-base mismatches variant spacer producing a relatively high proportion of incomplete-edited mutants. Although there may be other combinations of double-base mismatches that can produce a higher proportion of incomplete-edited mutants, we think that the M8 + M15 double-base mismatches variant spacer is adequate for the practical application of creating incomplete-edited mutants of TEGs.

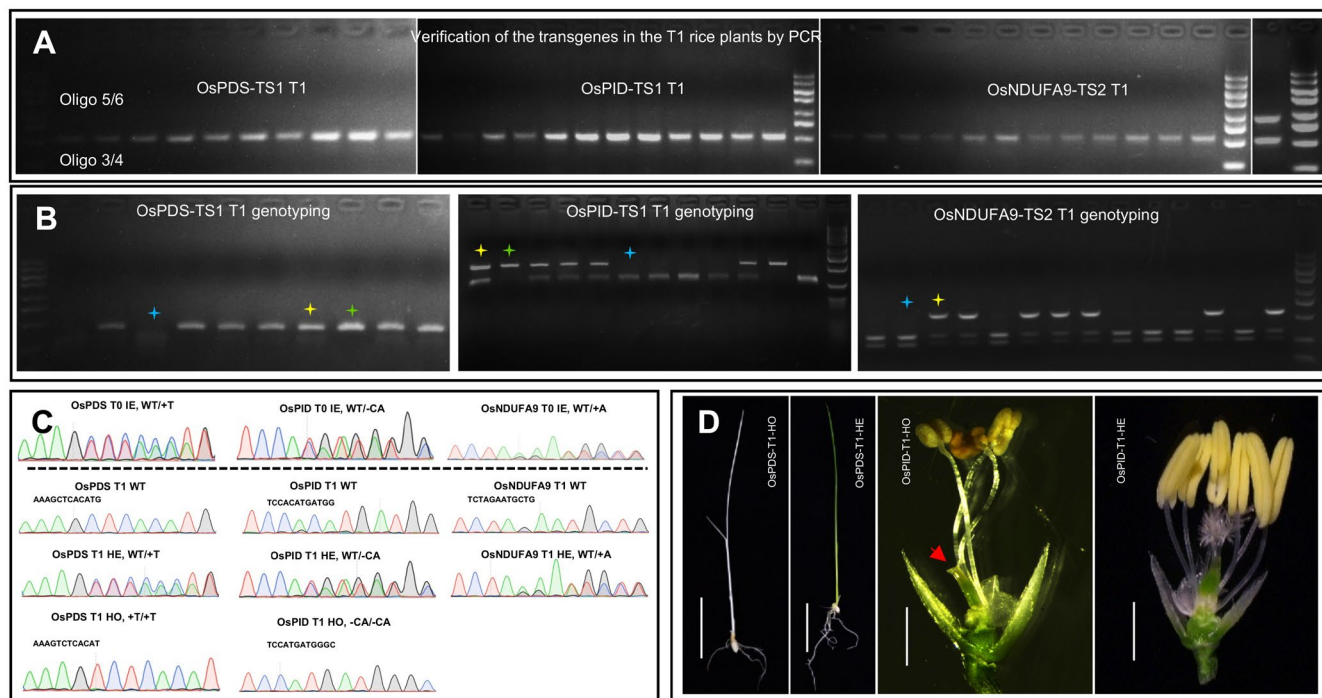


FIGURE 3 | Transmission analysis of incomplete-edited mutants. (A) Transgenic positive identification of T1 plants derived from T0 incomplete-edited mutants. ‘Oligo 3/4’ refers to the primer pair used for checking the quality of rice genomic DNA. ‘Oligo 5/6’ represents the primer pair used for detecting T-DNA. (B) Genotyping analysis of the progeny derived from T0 incomplete-edited mutants of *OsPDS*, *OsNDUFA9* and *OsPID*. ‘Green star’ refers to homozygous mutation. ‘Yellow star’ means heterozygous mutation. ‘Blue star’ indicates the wide type. (C) Representative sanger sequencing results of T0 plants with incomplete-edited mutations and their progeny of different genotypes. ‘IE’ refers to incomplete-edited mutation. ‘HO’ represents homozygous mutation. ‘HE’ means heterozygous mutation. ‘WT’ refers to the wide-type. (D) The phenotypes of T1 mutants of *OsPDS* and *OsPID*. Homozygous mutant of *OsPDS* exhibits albino phenotype, heterozygous mutant appears green. Scale bar, 2 cm. Homozygous mutant of *OsPID* gene exhibits absent stigmas (red arrowhead). Scale bar, 0.2 cm. Heterozygous mutant shows normal as wild-type. ‘HO’ refers to homozygous mutation. ‘HE’ means heterozygous mutation.

2.3 | TKC-Driven Generation of Stable and Heritable Heterozygous Mutants

To confirm that the incomplete-edited mutants produced by this method are heritable, we selected T0 plants with incomplete-edited mutations at the *OsPDS*-TS1, *OsPID*-TS1 or *OsNDUFA9*-TS2 target sites for genetic analysis and examined the phenotypes and genotypes of their T1 progeny (Figure 3). As expected, all T1 plants were transgenic negative (Figure 3A), indicating that the *TKC* vector autonomously removed the transgenic elements during propagation, which also prevents the incomplete-edited target site from being further edited into homozygous or biallelic mutations. Subsequently, we analyzed the mutation types of the target genes (Figure 3B) and sequenced some plants for verification (Figure 3C). The T1 progeny of *OsPDS*-TS1 and *OsPID*-TS1 had three genotypes: homozygous, heterozygous and wild type (Figure 3C). The T1 progeny of *OsNDUFA9*-TS2 had only two genotypes: heterozygous and wild type (Figure 3C). As expected, the homozygous mutant of *OsPDS* exhibited an albino phenotype, but the heterozygous mutant appeared green. The homozygous mutant of *OsPID* exhibits absent stigmas. However, the heterozygous *ospid* mutant shows normal as wild type (Figure 3D). This is consistent with previous studies, as homozygous mutations in *OsNDUFA9* lead to embryo lethality (Hu, Miller, et al. 2018; Hu, Tian, et al. 2018). Therefore, the

incomplete-edited mutants obtained by our method can be stably inherited and can be propagated through heterozygous mutations in subsequent generations.

2.4 | Tripartite Spacers Cocktail via *Agrobacterium* Co-Transformation Maximises Incomplete Editing for Target Genes

Although we have identified that single-base mismatch at position M11 or M17 was suitable for THSM targets, and dual-base mismatches at positions M8 + M15 were suitable for TLSM targets, without prior testing, we cannot determine whether the selected target site is a THSM or TLSM target for any given gene. Therefore, we tested a simple cocktail method: we co-infected rice calli with *Agrobacterium* carrying *TKC* vectors with three types of spacers (G1, M11 and M8 + M15), which we named the *TKC*-M Cocktail (*TKC*-MC) method (Figure 4A). In this way, G1 ensures the basic proportion of incomplete-edited mutants for THSM targets. M8 + M15 enables TLSM targets to produce a high proportion of incomplete-edited mutants. To verify the feasibility of our method, we selected two seedling albino lethal genes, *OsEF-Tu* (Xu et al. 2024) and *OsRPL21c* (Lin et al. 2015), for testing. We constructed *TKC* vectors with three types of spacers (G1, M11 and M8 + M15), and then

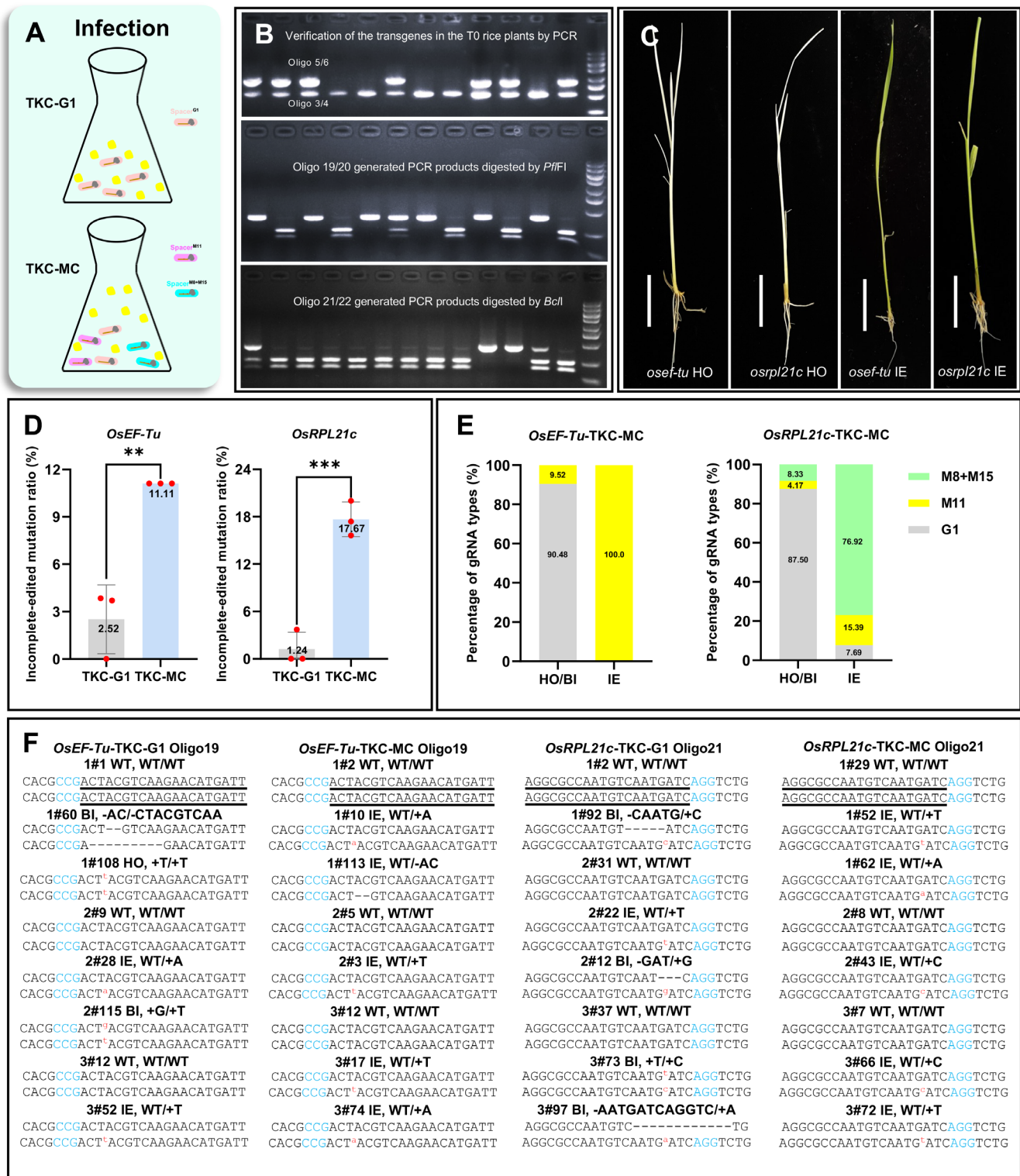


FIGURE 4 | Legend on next page.

co-infected rice callus with *Agrobacterium* carrying these vectors (Figure 4A). After verification of transgenic positivity and identification of target gene mutations (Figure 4B), we successfully obtained two types of mutants: green incomplete-edited mutants and albinistic knockout mutants (Figure 4C). The data showed that the proportion of incomplete-edited mutants obtained through TKC-M was significantly higher than that obtained through TKC-G1 (single transformation with

TKC vector containing G1 spacer) (Figure 4D, Table S3). For *OsEF-Tu*, the proportion of incomplete-edited mutant plants obtained through TKC-G1 was only 2.52%, while that produced through TKC-MC was 11.11% (Figure 4D, Table S3). For *OsRPL21c*, the ratio of incomplete-edited mutants produced through TKC-MC was 17.67%, while that produced by TKC-G1 was only 1.24% (Figure 4D, Table S3). This indicates that the TKC-MC method can effectively produce a high proportion

FIGURE 4 | Incomplete-edited mutants ratios increased in TKC-MC with mixed *Agrobacterium* cocktail. (A) Comparison between routine rice tissue culture infection and mixed infection. ‘TKC-G1’ refers to routine infection of *TKC* construct containing Spacer^{G1} without mismatch. ‘TKC-MC’ means mixed rice tissue culture infection of *TKC* constructs containing Spacer^{G1} (without mismatch), Spacer^{M11} (with M11 mismatch), and Spacer^{M8+M15} (with M8 and M15 mismatches). (B) The transgenic positive T0 plants were identified by PCR amplifications. ‘Oligo 3/4’ refers to the primer pair used for checking the quality of rice genomic DNA. ‘Oligo 5/6’ indicates the primer pair used for detecting T-DNA. ‘Oligo 19/20’ means the primer pair used for genotyping of T0 plants containing OsEF-Tu-TS1 targeting to *OsEF-Tu*. ‘Oligo 21/22’ represents the primer pair used for genotyping of T0 plants carrying OsRPL21c-TS1. (C) The phenotypes of the homozygous mutants of *OsEF-Tu* and *OsRPL21c*. Both homozygous mutants of *OsEF-Tu* and *OsRPL21c* exhibit albino phenotypes, whereas the incomplete-edited mutants remain green. ‘HO’ refers to homozygous mutants. ‘IE’ represents incomplete-edited mutants. Scale bar, 2 cm. (D) Comparison of incomplete-edited mutation ratios across different transformation events. Incomplete-edited mutation ratio = incomplete-edited mutants/number of transgene plants. TKC-G1 represents the transformation event of *TKC* construct without mismatch in spacer to *OsEF-Tu* or *OsRPL21c*. TKC-MC means the cocktail transformation event of *TKC* constructs with G1, M11 and M8 + M15 mismatched spacers targeting to *OsEF-Tu* or *OsRPL21c*. ** $p < 0.01$, *** $p < 0.001$ (t -test). (E) Statistics of gRNA type integrated into the genome of mutants from mixed transformation events. ‘IE’ refers to incomplete-edited mutants. ‘HO/BI’ means homozygous mutants/biallelic homozygous mutants. ‘G1’ indicates the gRNA without spacer mismatch. ‘M11’ represents the gRNA with M11 spacer mismatch. ‘M8 + M15’ refers to the gRNA with M8 and M15 spacer mismatch. (F) The representative sequencing results of T0 plants generated from OsEF-Tu-TKC-G1, OsEF-Tu-TKC-MC, OsRPL21c-TKC-G1 and OsRPL21c-TKC-MC. The PAM sites are marked in light blue. ‘WT’ refers to wild-type Zhonghua 11 plants. ‘IE’ means incomplete-edited mutants. ‘HO’ represents homozygous mutants. ‘BI’ indicates biallelic homozygous mutants. ‘-’ refers to deletion of one base pair. ‘a’, ‘t’, ‘c’ and ‘g’ in red and superscript represents an insertion of an ‘A’, ‘T’, ‘C’ and ‘G’, respectively.

of incomplete-edited mutants. To further demonstrate the reliability of our method, we analyzed the gRNA types in all T0 mutants from the TKC-MC (Figure 4E, Table S4). In the *OsEF-Tu*-TKC-MC, 90.48% of the gRNAs detected in the homozygous/bi-allelic mutants were the G1 spacer, with a small portion being M11, and none being M8 + M15 (Figure 4E, Table S4). In the incomplete-edited mutants, all detected gRNAs were the M11 spacer. For the *OsRPL21c*-TKC-MC, 87.50% of the spacers detected in the homozygous/bi-allelic mutants were G1, with a small portion being M8 + M15, and the least being M11 (Figure 4E, Table S4). In the incomplete-edited mutants, over 70% of the gRNAs were the M8 + M15 spacer, followed by M11, and the least being G1 (Figure 4E, Table S4). Furthermore, we confirmed the mutations of incomplete-edited and homozygous/bi-allelic mutants via Sanger sequencing (Figure 4F, Figure S3). This indicates that in the TKC-MC transformation events, the vast majority of incomplete-edited mutants were produced by *TKC* vectors carrying variant spacers with base mismatch(es) relative to the target site. In summary, in practical applications, for any essential gene, co-transforming rice with *Agrobacterium* carrying three *TKC* vectors with three types of spacers (G1, M11 and M8 + M15), respectively, can produce a high proportion of incomplete-edited mutants, efficiently creating heritable mutants of TEGs.

3 | Discussion

In this paper, by harnessing spacer-mismatch tuning within our Transgene Killer CRISPR (TKC) platform, we convert finely reduced Cas9 activity into stable TEG heterozygotes that remain editable in T0 yet escape further modification in progeny. By tailoring spacer mismatches on *TKC* vectors, we optimised genome editing of TEGs and devised the streamlined TKC-MC protocol. Sensitive targets yielded abundant incomplete-edited T0 plants with the M11 spacer, whereas insensitive targets responded best to the M8 + M15 spacer. Irrespective of locus sensitivity, co-infection with a 1:1:1 mixture of *Agrobacterium* harbouring G1, M11 and M8 + M15

TKC vectors routinely generated a high proportion of heterozygotes. Because *TKC* autonomously excises the transgene cassette (He et al. 2018), the desired mutations remain protected from further editing and are stably transmitted to subsequent generations.

CRISPR/Cas9 has streamlined mutant generation, yet its standard configuration with high-level expression of Cas9 and gRNA drives near-complete editing. Persistent nuclease activity converts every allele to homozygous or biallelic states (Ma et al. 2015). In 328 T0 rice plants, only 5.8% retained heterozygous mutations (Ma et al. 2015). This result is consistent with our previous research results (He et al. 2019; He, Zhu, et al. 2020). Therefore, TEG disruption causes lethality or sterility, leaving genome-wide mutant collections devoid of essential alleles (Chen et al. 2022). For example, the female-sterility gene *OsPID* was inaccessible until labor-intensive EMS mutagenesis yielded rare heterozygotes (He et al. 2019). Alternative strategies generated weak alleles via 3'-terminal edits or in-frame indels (Minkenberg et al. 2017; Zhang et al. 2024), which require extensive screening or prior functional knowledge and remain impractical for uncharacterised TEGs. Using sperm- or egg-cell-specific promoters with CRISPR/Cas shows promise for generating heritable heterozygous mutations in target genes (Xu et al. 2018; Zheng et al. 2020). However, it takes more time and labor to identify the transgene-free gene-editing mutants in T2 and beyond. Moreover, this approach risks continuous germline editing in T1 and followed generations, potentially introducing new mutations and destabilising allele transmission. Here, we introduce TKC-MC, a rapid, knowledge-independent pipeline that reliably generates heritable, incompletely edited TEG mutants.

Since some factors such as spacer-target binding affinity, intramolecular spacer secondary structure, spacer-gRNA scaffold interactions, chromosomal context of the target site, and other variables collectively influence gene editing efficiency, establishing a simple routine method to increase the proportion of incomplete-edited mutants remains challenging. Based on prior studies and our data, introducing deliberate base mismatch(es) between the spacer and target site represents a viable strategy.

To generalise common characteristics underlying target-specific variability, we categorised target sites into two types according to their tolerance for single-base mismatches: THSM and TLSM (Figure 5). For THSM sites, single-base mismatches effectively increase the frequency of incomplete edits (Figure 1E). Based on the analysis of incomplete-edited mutants from single-base mismatches across three THSM-type target sites, spacer variants M10, M11, M12 and M17 are optimal, achieving ratios of 15.56%–45.16% (Figure 5). For TLSM-type sites, single-base mismatches often maintain editing efficiency but yield lower ratios of incomplete edits (Figure 1D). Consequently, we assessed double-base mismatches in two TLSM-type target sites and found that spacers M8+M15, M9+M15 and M10+M15 are most effective, with incomplete-edited ratios ranging from 32.76% to 61.11% (Figure 5). The M8+M15 spacer was able to result in a higher ratio of incomplete-edited mutations for the three tested targets (Figure 2C).

We therefore devised TKC-MC, a one-step protocol in which rice calli are co-infected with an equimolar mixture of *Agrobacterium* strains carrying TKC vectors harbouring M11, M8+M15 or control G1 spacers. This approach efficiently generates both homozygous and heterozygous mutants in a single transformation. The frequency of incomplete-edited mutants from TKC-MC events reached 11.11%–20.00%, markedly higher than the 0.00%–3.85% from TKC-G1 controls (Figure 5). Thus, TKC-MC enables direct generation of heritable mutants for any locus, irrespective of whether homozygous mutants are lethal or of the target's tolerance to single-base mismatches. This saves considerable trial-and-error time and supplies ready material for essential genes research. Importantly, beyond CRISPR/Cas9, this framework is readily portable to Cas12a which harbour a mismatch-sensitive core region (Wang et al. 2020). By categorising targets into sensitivity classes and identifying optimal mismatch positions within each category, this approach can be adapted to Cas12-based systems and other RNA-guided nucleases (Xu et al. 2017; Strohkendl et al. 2018; Wang et al. 2025). In summary, TKC-MC effectively addresses the challenge of creating inheritable mutants for homozygous lethal or sterile genes, particularly female-sterility genes. It facilitates the development of a comprehensive, inheritable rice mutant library and provides a crucial technical foundation for the functional study and extensive utilisation of these genes.

4 | Materials and Methods

4.1 | Plant Materials and Growth Conditions

Oryza sativa L. ssp. *japonica* cv. Zhonghua 11 (ZH11) was used in this study. All rice materials were grown in an experimental field in Wuhan during the natural growing season. The spacing between every two plants was 26.6 cm × 16.7 cm in the field.

4.2 | Target Design and Spacer Mismatch Engineering

Spacer sequences of gRNA were computationally predicted against the genome of *O. sativa* L. ssp. *japonica* cv. Zhonghua 11 (ZH11) using the CRISPR-P v2.0 web tool (<http://crispr.hzau.edu.cn/CRISPR2/>).

Candidates were preferentially selected when a restriction endonuclease recognition site overlapped the 3-bp region immediately upstream of the PAM. Site-directed single-nucleotide mismatches (A, T, C or G) were subsequently introduced into the spacer sequence, while avoiding any new off-target sites predicted by CRISPR-P (<http://crispr.hzau.edu.cn/CRISPR/>). All spacer sequences and their corresponding mismatches are provided in Table S5.

4.3 | TKC Vector Construction

All CRISPR/Cas9 constructs were constructed from the *TKC-D* vector which contains an *OsU6c* expression cassette (Yang et al. 2022). First, the *TKC-D* vector was digested with *Spe* I. Then, the spacers paired to targets of *OsPDS* (LOC_Os03g08570), *OsNDUFA9* (LOC_Os02g08168), *OsPID* (LOC_Os12g42020), *OsEF-Tu* (LOC_Os02g38210) and *OsRPL21c* (LOC_Os02g15900) were inserted into the *TKC-D* vector at the *Spe* I site by using Gibson assembly (Gibson et al. 2009), respectively. The primers were designed as follows: The forward primer was 5'-GNNNNNNNNNNNNNNNNgttttagctagctagaatagcaagta-3'. The reverse primer was 5'-MMMMMMMMMMMMMMMMMMMMMMMMMCAacctgagcctcagcgcagc-3'. 'M' represents the reverse complement sequence of 'N' in the forward primer (Table S6). Positive clones were verified by colony PCR using the Oligo 1 and Oligo 2 primer pair, followed by Sanger sequencing with Oligo 9. All primer sequences are provided in Table S6. The detail of the vector assembly proceeds in three tightly controlled steps. First, 1 µg of TKC-D plasmid is digested with *Spe* I (NEB, 1 U/µg, 37°C, 1 h) to generate linear fragments, which are purified with a DNA cleanup kit and eluted in 50 µL of 10 mM Tris-HCl (pH 8.0). Second, 100 µM forward and reverse primers are mixed 1:1, heated to 99°C for 10 min, and cooled to 25°C at 0.1°C/s to form double-stranded adapters. Third, a three-step Gibson reaction is performed: (i) 50 ng of linear vector is pre-incubated with 2× Gibson Master Mix (NEB) in a 1:2 volume ratio at 50°C for 20 min for 5'-exonuclease-mediated end polishing; (ii) annealed adapters are added (final molar ratio insert: vector = 3:1) and incubated at 50°C for 5 min to complete homologous recombination; (iii) the reaction is quenched on ice for 2 min and immediately used for chemical or electroporation transformation into *E. coli*. The following steps were the positive clones' identification and Sanger sequencing.

4.4 | Rice Genetic Transformation

All CRISPR/Cas9 constructs were verified by Sanger sequencing prior to transformation into *Agrobacterium tumefaciens* strain EHA105. For normal transformation, TKC constructs were introduced into calli of wild-type rice ZH11 using a previously described protocol (Hiei et al. 1994). For the TKC-MC transformation, each of the three constructs containing G1, M11 and M8+M15 spacers was individually introduced into EHA105. During tissue culture, each *Agrobacterium* suspension was prepared separately. After individual quantification of the concentration of each *Agrobacterium* suspension, the *Agrobacterium* suspensions were pooled in equal volumes to create a uniform cocktail for rice callus infection.

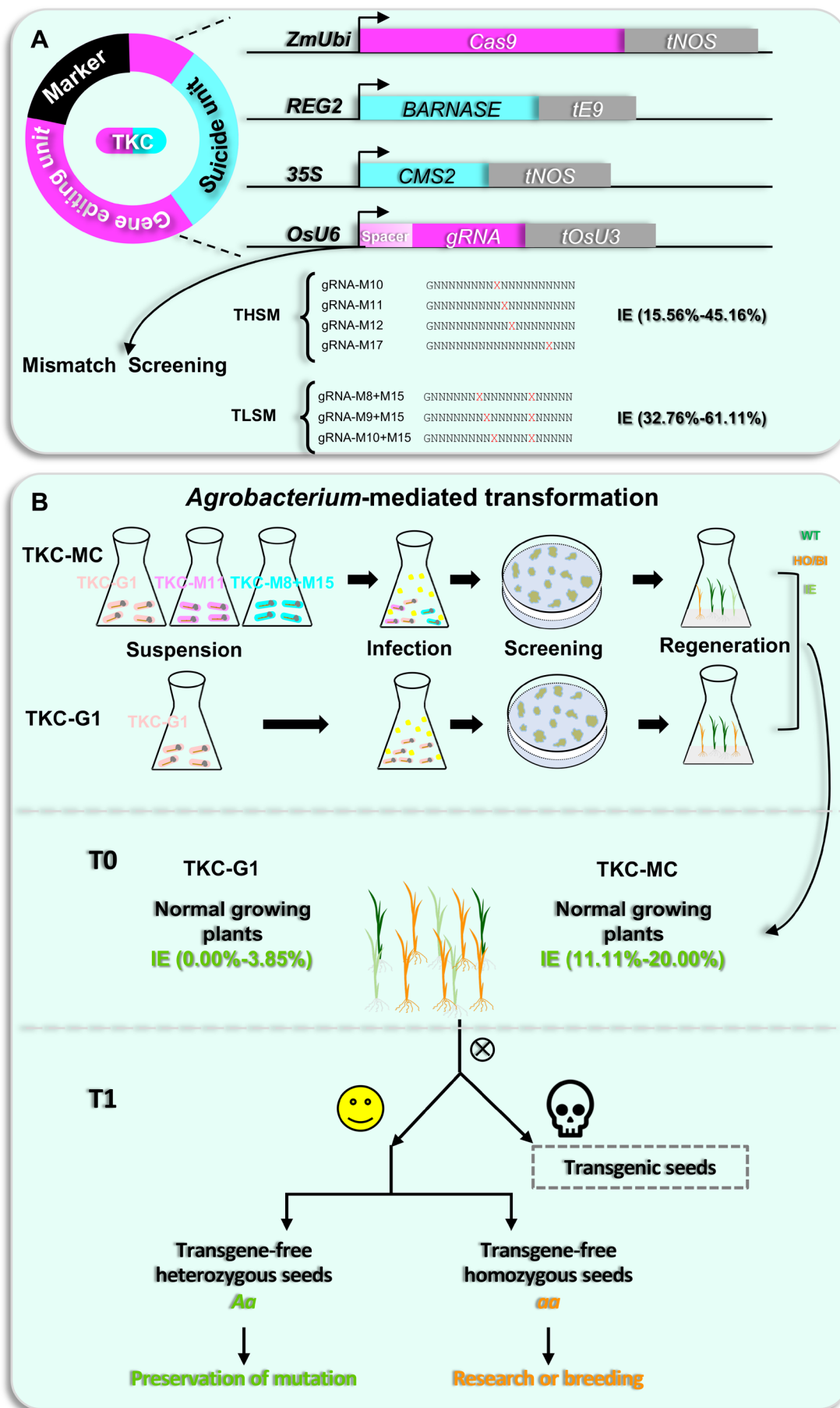


FIGURE 5 | Legend on next page.

FIGURE 5 | Schematic diagram of the mismatched spacer screening process and workflow for TKC-MC editing. (A) Schematic diagram of the mismatched spacer screening process. Vectors containing mismatched spacers in the *TKC* vector backbone. Different types of base mismatches were systematically introduced into spacers targeting various genomic loci for screening purposes. For targets exhibiting significant sensitivity to single-base mismatch (THSM), we identified spacers containing mismatch at positions 10–12 or 17 that generated a higher proportion of incomplete-edited mutants, with incomplete-edited mutation ratio ranging from 15.56% to 45.16%. For targets demonstrating lower sensitivity to single-base mismatch (TISM), double-base mismatch was required within the corresponding spacer region. Spacers containing the double-mismatch combinations M8 + M15, M9 + M15 and M10 + M15 proved effective in generating more incomplete-edited mutants, with incomplete-edited mutation ratio ranging from 32.76% to 61.11%. (B) Schematic diagram of the workflow for TKC-MC editing. For any gene whose sensitivity to single-base mismatch remains unknown, we adopted a TKC-MC co-transformation strategy. *Agrobacterium tumefaciens* strains harbouring the following *TKC* constructs were mixed and co-infiltrated into rice callus: TKC-G1 (control); TKC-M11 (single-base mismatch); TKC-M8 + M15 (double-base mismatches). Following callus screening, regenerated plants exhibited incomplete-edited mutation ratio of 11.11%–20.00%. These incomplete-edited mutant plants were grown to maturity under standard conditions, and seeds were harvested. Analysis of T1 progeny confirmed the absence of transgenic elements in all lines. Homozygous mutants are suitable for direct use in functional studies or breeding programs. Heterozygous mutants can be utilised to maintain and propagate the mutation.

4.5 | T0 Transgene Verification and Genotyping

First, all T0 transgenic plants were identified by PCR using the primer pair Oligo 5 and Oligo 6 to detect the presence of T-DNA. The primer pair Oligo 3 and Oligo 4 was used to check the quality of rice genomic DNA. For target gene genotyping, PCR plus restriction endonuclease digestion was performed. The PCR products were digested with specific restriction enzymes and analyzed on 1.5% (w/v) agarose gels. The primer pairs and corresponding restriction endonucleases were as follows: OsPDS-TS1 (Oligo 11/Oligo 12, *Hind* III), OsPDS-TS2 (Oligo 13/Oligo 14, *Afl* II), OsNDUFA9-TS1 (Oligo 7/Oligo 8, *Eco*N I), OsNDUFA9-TS2 (Oligo 15/Oligo 16, *Xba* I), OsPID-TS1 (Oligo 17/Oligo 18, *Pfl*M I), OsEF-Tu-TS1 (Oligo 19/Oligo 20, *Pfl*F I) and OsRPL21c-TS1 (Oligo 21/Oligo 22, *Bcl* I). Wild-type DNA is fully digested. Homozygous DNA is undigested. Incomplete-edited/heterozygous mutants show partial digestion. The primer sequences are listed in Table S6.

4.6 | Transmission Analysis of T0 Incomplete-Edited Alleles

Incompletely edited mutants from OsPID-TS1, OsNDUFA9-TS2 and OsPID-TS1 transformation events were selected for generational analysis. First, all T1 plants derived from these incomplete-edited mutants were identified by PCR using primer pair Oligo 5 and Oligo 6 to detect the presence of Cas9. The primer pair Oligo 3/Oligo 4 was used to assess genomic DNA quality. Genotyping of T1 mutants for OsNDUFA9-TS2 and OsPID-TS1 followed the same protocol as for T0 mutants. For T1 plants from OsPDS-TS1, PCR products amplified with primer pair Oligo 23/Oligo 12 were digested with *Alu* I. Sanger sequencing was performed on T1 plants with distinct genotypes to determine whether their mutation profiles were conserved or differed from those of the parental T0 plants. The primer sequences are listed in Table S6.

4.7 | Rice Stigma Phenotyping

Rice panicles were harvested at the late booting stage (approximately 1–2 days before anthesis). Spikelets containing unopened florets were selected and carefully excised from the central

rachis using fine forceps. The florets were placed on the stage of a stereomicroscope. After adjusting the field of view, the palea and lemma of each floret were carefully removed using forceps while observing through the microscope eyepieces. The remaining tissue was positioned in the center of the field of view to expose the stigma for observation and imaging.

Author Contributions

M.X. and Y.H. conceived the idea and wrote the first draft of the manuscript. M.X., L.Y., Z.Z., H.C., D.W., M.Z., Z.Z. and Y.Z. conducted the experiments. M.X. analyzed the data. M.X., L.X. and Y.H. contributed to manuscript revision. Y.H. supervised the study, and revised the manuscript. All authors contributed to the article and approved the submitted version.

Acknowledgements

We apologise to those whose work we were unable to cite due to space and reference limitations. We are deeply grateful to Prof. Yunde Zhao from the Department of Cell and Developmental Biology, University of California San Diego and Prof. Lanqin Xia from the Institute of Crop Sciences, Chinese Academy of Agricultural Sciences for their insightful comments and suggestions. This work is partly supported by the Biological Breeding-Major Projects (2023ZD04074), the National Natural Science Foundation of China (32200335 and U21A20207), the Youth Innovation Program of the Chinese Academy of Agricultural Sciences (Y2023QC39), the Nanfan special project of CAAS (YBXM2504 and YBXM2505) and the Earmarked Fund for the China Agriculture Research System (CARS-01).

Funding

This work was supported by Biological Breeding-Major Projects (2023ZD04074), Earmarked Fund for China Agriculture Research System (CARS-01), Nanfan Special Project of CAAS (YBXM2504 and YBXM2505), Youth Innovation Program of Chinese Academy of Agricultural Sciences (Y2023QC39) and National Natural Science Foundation of China (32200335 and U21A20207).

Conflicts of Interest

The authors declare no conflicts of interest.

Data Availability Statement

All data generated during this study is included in this published article and its [Supporting Information](#) files.

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Supporting Information

Additional supporting information can be found online in the Supporting Information section. **Figure S1:** The sanger sequencing results of T0 transgenic plants edited by TKC construct with single mismatched spacers. **Figure S2:** Mutation forms of T0 plants edited with multi-mismatch TKC constructs. **Figure S3:** The sanger sequencing results of T0 plants generated from TKC-G1 and TKC-MC at target genes of *OsEF-Tu* and *OsRPL21c*. **Table S1:** Mutation ratios and incomplete-edited mutation ratios of gRNAs with single-base mismatch of *OsNDUFA9*, *OsPDS* and *OsPID*. **Table S2:** Mutation ratios and incomplete-edited mutation ratios of gRNAs with multiple-base mismatches of *OsNDUFA9*, *OsPDS* and *OsPID*. **Table S3:** Incomplete-edited mutation ratios of single transformation (TKC-G1) and mixed transformation (TKC-MC) of *OsEF-Tu* and *OsRPL21c*. **Table S4:** Percentage of gRNA types in homozygous mutants or incomplete-edited mutants from TKC-Ms events targeting to *OsEF-Tu* and *OsRPL21c*. **Table S5:** The target sequences used in CRISPR/Cas9 gene editing by mismatched spacers. **Table S6:** Primer sequences used in this study.