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REVIEW

Technological breakthroughs in generating transgene-free and genetically stable CRISPR-edited plants

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Abstract CRISPR/Cas9 gene-editing technologies have been very effective in editing target genes in all major crop plants and offer unprecedented potentials in crop improvement. A major challenge in using CRISPR gene-editing technology for agricultural applications is that the target gene-edited crop plants need to be transgene free to maintain trait stability and to gain regulatory approval for commercial production. In this article, we present various strategies for generating transgene-free and target gene-edited crop plants. The *CRISPR* transgenes can be removed by genetic segregation if the crop plants are reproduced sexually. Marker-assisted tracking and eliminating transgenes greatly decrease the time and labor needed for identifying the ideal transgene-free plants. Transgenes can be programed to undergo self-elimination when *CRISPR* genes and suicide genes are sequentially activated, greatly accelerating the isolation of transgene-free and target gene-edited plants. Transgene-free plants can also be generated using approaches that are considered non-transgenic such as ribonucleoprotein transfection, transient expression of transgenes without DNA integration, and nano-biotechnology. Here, we discuss the advantages and disadvantages of the various strategies in generating transgene-free plants and provide guidance for adopting the best strategies in editing a crop plant.

Keywords CRISPR, Transgene-free, Marker-assisted selection, TKC, Cas9, gene editing

INTRODUCTION

The CRISPR genome editing technology has enabled efficient modifications of target DNA in living cells (Cong et al. 2013; Mali et al. 2013), providing powerful tools for developing cures for diseases and for crop improvement. Editing a gene in vivo by CRISPR only requires three components (Gasiunas et al. 2012; Jinek et al. 2012): a programmable nuclease such as Cas9, a guide RNA (gRNA), and a protospacer adjacent motif (PAM) in close proximity to the target DNA. The Cas9 and gRNA complex binds to the target DNA, which is complementary to part of the gRNA molecule. Subsequently, Cas9 generates a double-stranded break within the target sequence, providing opportunities for editing the target sequence through DNA-repair pathways.

It is straightforward to produce Cas9 and gRNA molecules in vivo using cellular transcription and translation machinery (Cong et al. 2013; Mali et al. 2013). Because of its simplicity and effectiveness, CRISPR has succeeded in editing genes in virtually every transformable organism (Char and Yang 2019; Cong et al. 2013; Mali et al. 2013; Mao et al. 2013; Miao et al. 2013; Schmidt et al. 2019). However, there are still major challenges ahead in terms of commercial applications of CRISPR technology in medicine and in agriculture. Interestingly, using CRISPR gene-editing

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technologies in crop improvement and in medical applications faces rather different challenges. For medical applications, the main concern is off-target effects (Akcakaya et al. 2018; Cullot et al. 2019; Kempton and Qi 2019; Zuo et al. 2019), which may cause serious unintended health consequences. Medical applications of CRISPR technology are generally involved in editing somatic cells. Editing germline cells in human and generating human individuals with transmissible mutations are ethically and legally not permitted at present or in the near future (Musunuru et al. 2017; Winblad and Lanner 2017). In contrast, crop improvement and plant breeding require the generation of plants with heritable traits. The off-target effects of CRISPR gene-editing technology in plants are also a concern, but any mutations generated by off-target effects can be easily removed through genetic crosses (Tang et al. 2019). It is also feasible to simply select the individuals that do not contain any off-target mutations. A main challenge in using CRISPR in agriculture is to obtain plants that are transgene free and that have been edited with stably transmissible traits (Gao and Zhao 2014). Any commercially viable target gene-edited crop plants need to be transgene free for several reasons. First, farming plants that contain a *CRISPR* gene-editing construct is potentially hazardous to the environment. Releasing *CRISPR*-containing pollen/seeds to the environment is not acceptable to the public and would make it very difficult to gain approval from government regulatory agencies (Callaway 2018; Huang et al. 2016). Second, traits in crops need to be stable and the presence of the *CRISPR* constructs makes it difficult to assess the stability and penetrance of the phenotypes because the CRISPR enzyme and guide RNA can continue to edit the existing targets or even off-targets (Gao and Zhao 2014).

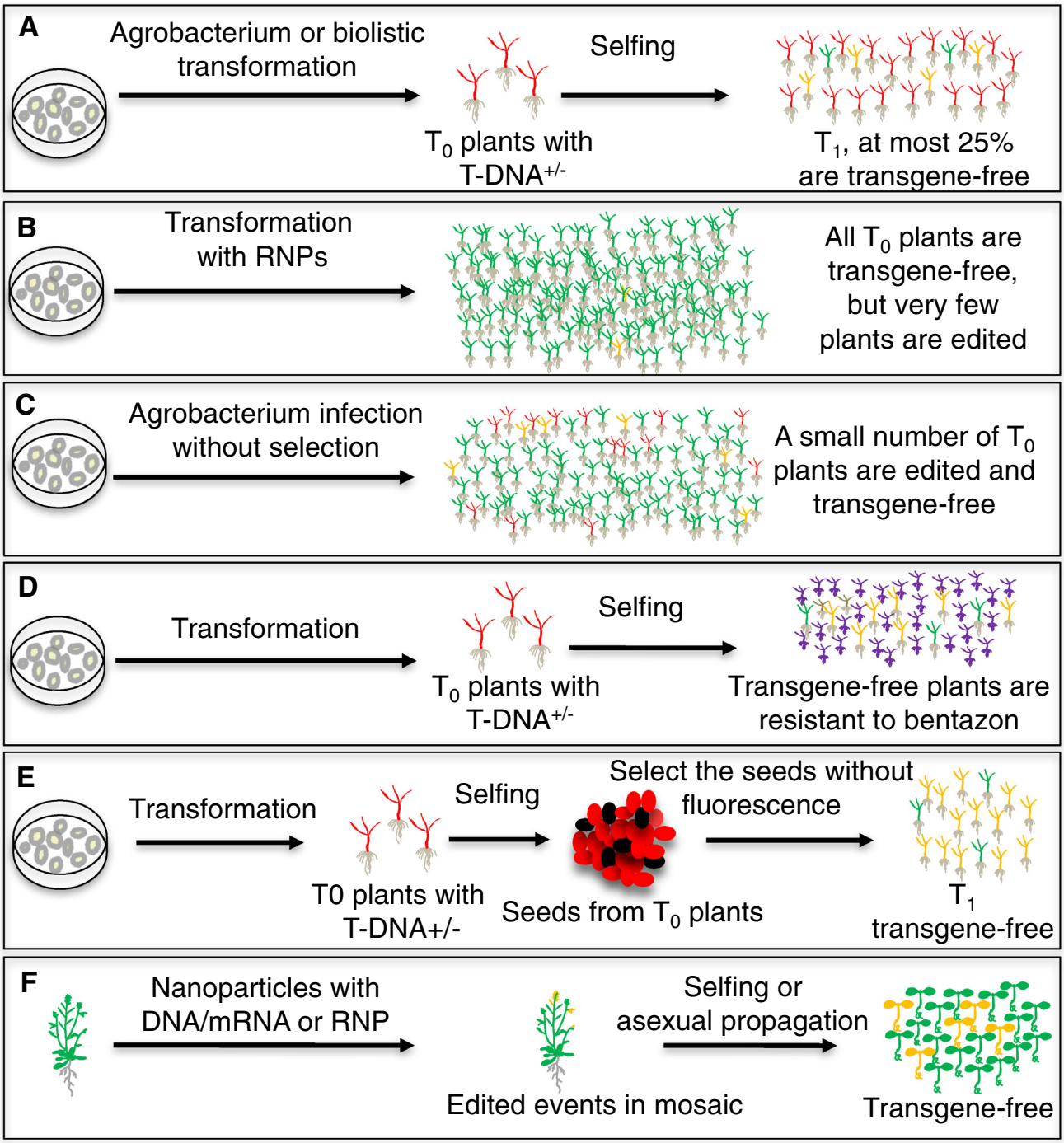
Because the absence of any transgenes in gene-edited plants is a prerequisite for commercialization of any CRISPR-edited plants with stable valuable traits, several strategies have been employed to generate CRISPR-edited plants that do not contain *CRISPR* constructs and other transgenes. The strategies include non-transgenic approaches (Luo et al. 2015; Woo et al. 2015), avoiding transgene integration(Chen et al. 2018), maker-assisted tracking and selecting plants with/without transgenes (Gao et al. 2016; He et al. 2017; Liu et al. 2019), and self-elimination of transgenes after plants are edited (He et al. 2018, 2019). The goal is to generate transgene-free and gene-edited crop plants with greatly reduced costs of labor and time. In this article, we describe the recent innovations that have improved the effectiveness in isolating CRISPR-edited and transgene-free plants.

ELIMINATION OF CRISPR CONSTRUCTS BY GENETIC SEGREGATION

The CRISPR gene-editing components including a Cas9-expression cassette, a gRNA-producing cassette, and selection markers such as a kanamycin-resistant gene are placed in a single plasmid, which is subsequently introduced into plant cells through either *Agrobacterium*-mediated transformation (Clough and Bent 1998; Hiei et al. 1994) or biolistic (gene gun) transformation (Sanford 1990) (Fig. 1A). The transgenes are integrated into plant chromosomes and are able to produce the CRISPR enzyme and gRNA for target gene editing. Some of the first-generation plants (T0) are edited at target loci and the majority of the T0 plants contain the *CRISPR* transgenes (Fig. 1A). The transgene locus is usually heterozygous in T0 plants (Fig. 1A). Both the transgene locus and the edited locus segregate according to Mendelian genetics in the progeny of T0 plants. A quarter of the T1 plants (progeny from the first generation T0 plants) is transgene free if only one copy of the transgenes was inserted into the genome (Fig. 1A). It is known that both *Agrobacterium*-mediated transformation and gene gun methods often lead to more than one insertion of the transgenes (Jeon et al. 2000; Lowe et al. 2009). Therefore, the actual probability of obtaining transgene free and edited plants among the progeny of T0 plants is much lower than 25%. Moreover, if the transgene locus and edited locus are linked, elimination of the transgenes by genetic segregation would become very difficult. Backcrossing of the T0 plants to wild-type plants can help eliminate off-target/background mutations and the transgenes, but it takes an extra generation to reach the goal of obtaining transgene-free and edited plants. Overall, isolation of transgene-free plants from the progeny of T0 plants or from backcrossed populations using genetic segregation is feasible and has been widely used, but it is labor and time intensive.

RIBONUCLEOPROTEIN (RNP) TRANSGENE-FREE GENE EDITING

Purified recombinant Cas9 and in vitro produced gRNAs can be reconstituted into a Cas9-gRNA RNP complex in vitro. The RNP complex can then be delivered via micro-injection in animal cells or by gene gun and other methods in plants (Woo et al. 2015). The RNP methods offer many advantages over plasmid DNA-based approaches, which depend on cellular machinery for the production of both Cas9 and gRNA. Some cell types may not be able to express adequate amount of CRISPR



◀**Fig. 1** The commonly used strategies for isolating transgene-free and CRISPR-edited plants. **A** Elimination of *CRISPR* transgenes by genetic segregation. The transgene locus is usually heterozygous in the first generation of transgenic plants (T_0). The transgenes in the next generation of plants segregate according to Mendelian genetics. The transgene-free and edited T_1 plants can be identified by PCR-based genotyping. **B** Gene editing by ribonucleoprotein (RNP). Nuclease and gRNA are reconstituted in vitro as RNP complexes, which can be delivered into plant cells using biolistic transformation or other methods. Both transformed and untransformed cells are able to regenerate into plant seedlings, making it extremely laborious to identify the edited plants, but all the T_0 plants are transgene free. **C** Gene editing by transient expression of a nuclease and gRNA. In the absence of a selection pressure, *Agrobacterium* infection can lead to the expression of transgenes without integrating the transgenes into chromosomes. Such events can lead to the generation of transgene-free and edited plants. **D** Drug-induced elimination of transgenes. The *CYP81A6* encodes an enzyme that metabolizes bentazon, an herbicide. Coupling *CYP81A6* RNAi with *CRISPR* components enables a selection for transgene-free and edited plants. **E** Fluorescence marker-assisted transgene elimination. The *mCherry* fluorescence marker is linked to the gene-editing components in the same plasmid. The marker allows selection of transgene-free seeds, greatly reducing the workload associated with growing plants and genotyping. **F** Nanotechnology-mediated gene editing. Nanoparticles coated with DNA, RNA, or RNP can deliver CRIPSR reagents into meristematic cells. This approach usually generates mosaic plants. The transgene-free and edited plants may be obtained by either sexual or asexual propagation from the edited tissues. The color scheme for plants used in the figure is wild-type (green), transgenic (red), transgene-free and edited (yellow), and dead plants (purple)

components. More importantly, the RNP method itself does not use any transgenes (Fig. 1B). Thus, the edited plants can obviously be classified as non-transgenic plants, rendering it an easier path for gaining regulatory approval.

The RNP complex is typically introduced into protoplasts, immature embryos, or calli using particle bombardment or transformation (Liang et al. 2017; Svitashev et al. 2016; Toda et al. 2019; Woo et al. 2015). Transformation of protoplasts is not difficult, but regeneration of a plant from a single protoplast is complicated and very few plant species are amenable to such a process (Lin et al. 2018). Therefore, few studies using RNP and protoplast transformation to generate edited plants have been reported. Bombardment of RNP complexes into immature embryos or calli is also able to achieve gene editing. However, unlike plasmid transformation that provides antibiotic/herbicide resistant markers, the RNP complex itself does not confer any selection markers if the edited plants do not have a visible phenotype (Fig. 1B). Without selection pressures, both the cells with RNP and cells without RNP will be able to regenerate into plantlets. In fact, the majority of plants generated from RNP bombardment of calli are not transformed and have not been edited, making it extremely laborious to isolate edited plants using PCR-based methods (Fig. 1B). So far, RNP methods have not been widely adopted by plant biology laboratories.

GENE EDITING BY *AGROBACTERIUM*-MEDIATED TRANSIENT EXPRESSION OF TRANSGENES

Agrobacterium-mediated plant transformation leads to insertion of transgenes into plant genomes. It is also well known that *Agrobacterium* can mediate transient transgene expression in plant cells (Amoah et al. 2001; Krenek et al. 2015; Li et al. 1992). Such a phenomenon can be used to transiently produce Cas9 and gRNA in plants for gene editing without integrating the transgenes into the genome, providing an approach for isolating transgene-free edited plants (Fig. 1C). Chen et al. used this strategy to successfully edit tobacco *PHYTOENE DESATURASE* (*PDS*) gene (Chen et al. 2018). A key difference between the Chen et al. transformation method and the traditional *Agrobacterium*-mediated transformation was that Chen et al. did not apply antibiotic selection to allow the survival of transiently transformed cells (Fig. 1C). Overall, Chen et al. achieved about 10% transgene-free and edited tobacco plants. Another main advantage of the transient methods is that no sexual segregation is needed for the elimination of transgenes. However, the T0 plants have at least three populations: plants with T-DNA insertions, transiently transformed plants, and untransformed plants (Fig. 1C). The ratios among the tree populations would vary depending on transformation conditions. It is still laborious to identify the desired plants without the transgenes using this method. Further engineering the Agrobacteria to reduce transgene integration and/or to increase the transient expression of transgenes in plant cells will greatly increase the efficiency of isolating transgene-free and edited plants.

DRUG-INDUCED ELIMINATION OF TRANSGENES

Transgenes may be negatively selected if the CRISPR constructs are properly designed and selection conditions are optimized. For example, the kanamycin-sensitive plants may be recovered if they are transferred to media containing no kanamycin at the proper time. Therefore, it is feasible to identify transgene-free plants from the progeny of T0 plants using antibiotic sensitivity screens. However, such a negative screen is tricky and may also lead to higher false positives.

Lu et al. took advantage of the RNA interference technology (RNAi) and developed a strategy that eliminates plants with *CRISPR* constructs in a drug-dependent manner (Lu et al. 2017) (Fig. 1D). The cytochrome P450 *CYP81A6* gene renders rice plants resistant to bentazon and sulfonylurea herbicides. Rice plants with a compromised *CYP81A6* are hypersensitive to bentazon. Lu et al. added an RNAi cassette that targets *CYP81A6* to the *CRISPR* plasmid. Such a construct allows editing of the target DNA and simultaneously knockdown of *CYP81A6.* The latter makes the plants hypersensitive to herbicide bentazon (Fig. 1D). Therefore, transgene-containing plants can be effectively eliminated at the T1 generation under the selection of bentazon (Fig. 1D). This method can greatly eliminate the labor required for selecting transgene-free plants. Although overexpression of *CYP81A6* in Arabidopsis can lead to bentazon resistance, it is not clear whether RNAi of *CYP81A6* can be adopted for selecting transgene-free and edited plants in other species.

FLUORESCENCE MARKER-ASSISTED TRANSGENE ELIMINATION

The most labor and time-intensive part of isolating transgene-free and edited plants is to grow the plants, collect leaf samples, prepare genomic DNA, and genotype by PCR. If there is a simple way to differentiate transgenic seeds from non-transgenic seeds, it can eliminate the need to grow the undesired progeny from T0 plants, thus greatly reducing the workload for obtaining transgene-free and edited plants. Gao et al. linked an *mCherry* fluorescence marker gene to *CRISPR* components in the same plasmid so that *mCherry* can be used as a proxy for transgenes (Gao et al. 2016). Moreover, Gao et al. placed the *mCherry* gene under the control of a strong seed promoter *At2S2*, allowing an easy identification of seeds with/without transgenes (Fig. 1E). The fluorescence marker-assisted CRISPR technology greatly simplified gene editing in Arabidopsis (He et al. 2017; Yu and Zhao 2019). Transgenic T1 seeds can be easily identified by the strong red florescence (Fig. 1E). Furthermore, the fluorescence intensity usually correlates with the expression levels of Cas9 and gRNA because they are linked in the same plasmid, providing a clue for selecting plants with high gene-editing efficiency. In the next generation, only transgene-free seeds are selected for determining whether the targeted sequences have been edited. Any gene-editing events identified in the non-transgenic plants from the T0 progeny are inherited from the previous generation. Therefore, they can be stably transmitted into future generations.

The fluorescence marker-assisted CRISPR technology can easily be extended to other plants. For example, high-throughput fluorescence sorting of rice seeds has been developed to assist the screening of plants without transgenic fragments (Chang et al. 2016). Moreover, some other natural color compounds can also be used as a marker to help select the transgene-free gene-edited plants (Liu et al. 2019). Even though marker-assisted identification of transgene-free plants reduces workload by at least 75%, the strategy still requires significant amounts of labor and time.

NANOTECHNOLOGY-MEDIATED GENE EDITING

CRISPR gene editing relies on the effective delivery of nucleases and gRNAs inside cells, but many plant species are not transformable at present. Therefore, transformation-independent gene editing will be very valuable to crop improvement. One of the approaches is to use nanoparticles as delivery vehicles for CRSIPR components (either DNA/RNA or RNP). Several types of nanomaterials including magnetic nanoparticles, carbon nanotubes (CNTs), and carbon dots have been tested for their potential in genetic engineering in plants (Bao et al. 2017; Demirer et al. 2019a; Doyle et al. 2019; Wang et al. 2016). Carbon nanotubes (CNTs) coated with bio-macromolecules (such as plasmid DNA) can passively pass through extracted chloroplasts and plant membranes without being degraded by cellular metabolic and degradation enzymes. It was demonstrated that diffusion-based biomolecule could be delivered into intact plants of several species including tobacco, musk, wheat, and upland cotton. However, it is not clear whether CNTs can deliver gene-editing reagents into plant cells to successfully edit target genes (Demirer et al. 2019b). Magnetic nanoparticles can be infiltrated into cotton pollen grains facilitated by magnetic force. The nanoparticle-infiltrated pollen grains are still viable and are able to pollinate cotton plants (Zhao et al. 2017). Nanoparticle-infiltrated cotton pollen grains successfully introduced marker genes such as β-glucuronidase into cotton, demonstrating that transformation-independent approaches of genetic engineering might be feasible in plants. Magnetic nanoparticles as a delivery vehicle of genome editing reagents appeared have not been adopted widely because no follow-up studies have been published.

Nanoparticles can potentially deliver gene-editing cargos to any plant cells including meristematic cells (Sanzari et al. 2019; Wang et al. 2019). Delivery of gene-

editing reagents through nanoparticles into meristematic cells can potentially generate chimerically edited plants (Fig. 1F). Transgene-free and edited plants may be regenerated from the edited tissue through tissue culture or from propagation of cuttings. A recent exciting report indicates that plasmid coated carbon dots can be delivered into plant cells by foliar application (spraying on) and that Cas9/gRNAs produced by this method successfully edited target genes (Doyle et al. 2019). This new method potentially can be extended to other plants, offering a simple, fast, and inexpensive method for editing plant genomes. Despite the potential of nano-biotechnology in gene editing in plants, much research including the safety of nanoparticles to human health and the environment is still needed.

SELF-ELIMINATION OF TRANSGENES: THE TKC TECHNOLOGY

Genetic segregation and marker-assisted (positive or negative selection) CRISPR for identifying transgene-free plants still require significant labor and time. Ideally, a CRISPR construct would undergo self-elimination after gene-editing tasks have been completed. Such a CRISPR design would greatly reduce the time and labor involved in isolating transgene-free and edited plants. We name the self-eliminating and Transgene Killer CRISPR system TKC technology (He et al. 2018, 2019) (Fig. 2). We designed two functional units for TKC technology: a gene-editing unit that includes nuclease and gRNA expression cassettes and a suicide unit that produces toxic proteins (Fig. 2A). The expression of the two units is temporally controlled and takes place sequentially. The gene-editing unit is activated first to release Cas9 and gRNA so that target genes are edited (Fig. 2B). After the target genes are edited, the suicide genes are programmed to trigger cell death (Fig. 2C). Consequently, only the transgene-free cells will survive, providing a positive- and self-selection for the transgene-free plants.

The gene-editing unit can be easily adapted from any previously reported CRISPR constructs (Gao et al. 2015). We used the maize *UBIQUITIN* promoter to drive *Cas9* expression and produced gRNAs using the rice *U6* promoter (He et al. 2018). Construction of the transgene-killer unit is slightly more nuanced. The unit should not be activated in somatic cells. Otherwise the toxic proteins will likely kill the T0 plants. We programmed the activation of the suicide unit during the reproductive phase of the T0 plants (Fig. 2C). Because the T-DNA insertion locus in T0 plants is usually heterozygous (Fig. 1A), we hypothesized that transgene-free gametes would be produced, whereas transgene-containing gametes can be killed if the suicide genes are activated during the right timeframe (He et al. 2018).

We chose the ribonuclease *BARNASE* from *B. subtilis* as one of the suicide genes (Hartley 1988) (Fig. 2A). BARNASE was previously shown to effectively kill plant cells when expressed inside cells (Mariani et al. 1990). Overexpression of the rice cytoplasmic sterility gene *CMS2* is known to cause cell death in pollen (Hu et al. 2013; Wang et al. 2013; Wang et al. 2006). Therefore, CMS2 may be used to kill transgene-containing pollens. We designed two suicide gene cassettes to ensure all the T0 progeny that contain a transgene would be eliminated (He et al. 2018). In the first suicide cassette, the *BARNASE* gene was under the control of the *REG2* promoter, which is active during the early rice embryo development (Sun et al. 1996) (Fig. 2A). The second suicide cassette used the *CaMV 35S* promoter to drive the rice cytoplasmic sterility gene *CMS2*, which can block the normal function of mitochondria during pollen development (Fig. 2A).

The TKC system automatically eliminates those plants that contain the CRISPR construct, but still enables the plants to undergo targeted gene editing before the transgenic DNA fragments are removed. During tissue culture and vegetative growth, Cas9 and gRNAs, which are driven by constitutively active promoters, are produced to enable targeted gene editing (Fig. 2B). Because *BARNASE* is not expressed in calli and during vegetative growth and CMS2 does not cause death of somatic cells, cells harboring the transgenes in T0 plants can be edited, but will not be killed. When the T0 plants reach the reproductive phase, the male gametophytes with the TKC construct are aborted due to the toxic effects of CMS2. Therefore, non-transgenic pollen grains are enriched while the transgene-containing pollen grains are eliminated. After fertilization, BARNASE will specifically kill the embryos that contain the transgenic DNA fragment, resulting in the complete elimination of transgenic progeny from T0 plants. Any resulting seeds from T0 plants will not contain transgenes. Some of survived progeny of T0 plants harbor edited events.

We selected the *OsLAZY1* (Li et al. 2007) to demonstrate proof-of-concept of the effectiveness of TKC in identifying transgene-free edited plants (He et al. 2018). Molecular analyses demonstrate that all the progeny from the independent T0 plants generated by TKC vectors were transgene-free whereas at least 75% of the offspring from T0 plants generated from traditional CRISPR vectors still had the CRISPR construct (He et al. 2018). Moreover, all the transgene-free T1 plants analyzed harbored the edited events in the target gene,

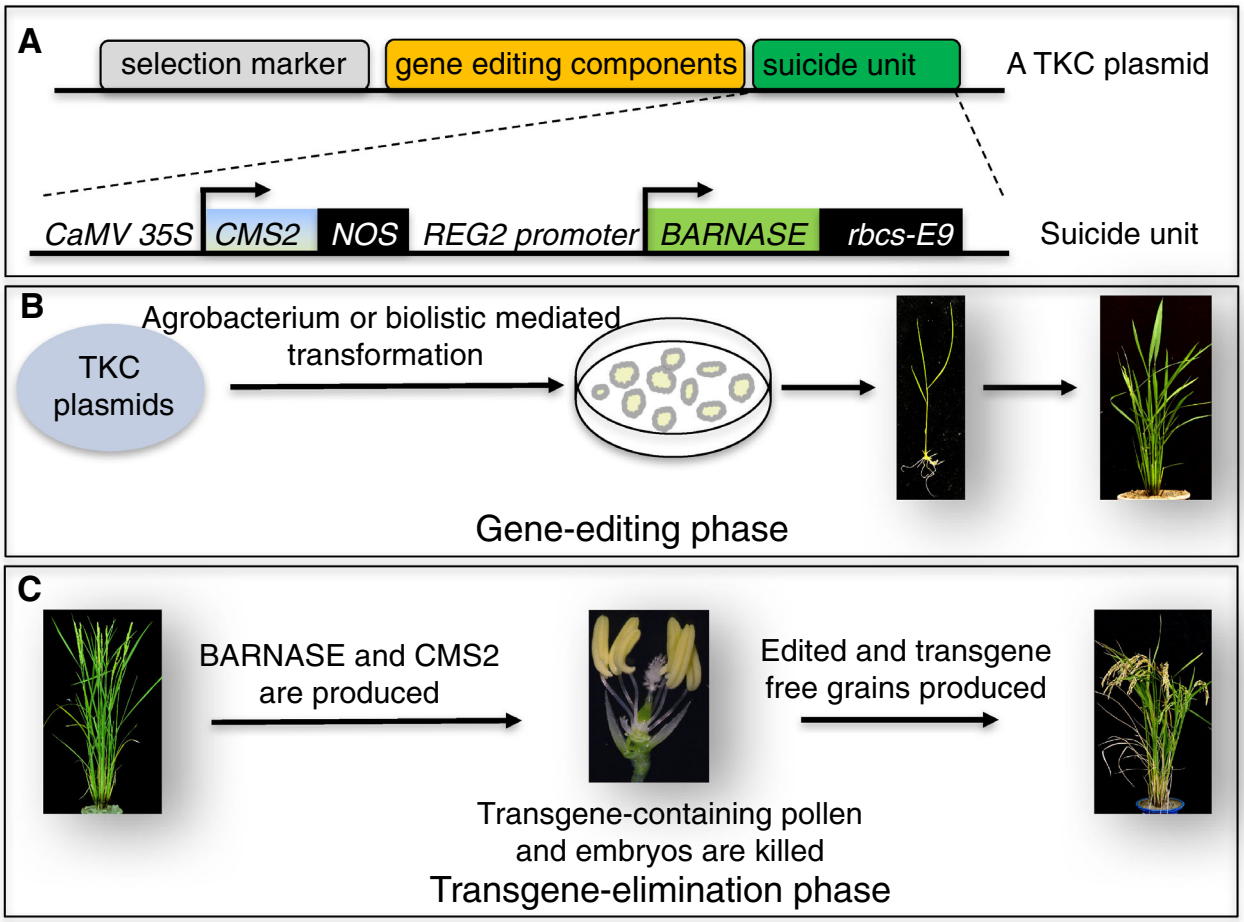


Fig. 2 Programmed suicide gene-mediated self-elimination of transgenes for accelerated identification of transgene-free and CRISPR-edited plants. **A** The key components of a *TKC* (*Transgene Killer CRISPR*) plasmid. The suicide unit is placed in the same plasmid with a selection marker and the *CRISPR* gene-editing components. The suicide unit is divided into two sub-units: the *CMS2* under the control *CaMV 35S* promoter kills any transgene-containing pollen grains; the *BARNASE* gene controlled by an early embryogenesis-specific promoter *REG2* causes death of embryos that harbor the transgenes. **B** The gene-editing phase of TKC. During tissue culture and vegetative growth, both nuclease and gRNA are expressed and the target genes are edited. During this phase, the suicide units are not activated or not toxic. **C** The transgene-elimination phase of TKC. At T_0 plants transition to the reproductive growth, many of the cells in the plants have already been edited. CMS2 and BARNASE cause death of pollen grains and embryos, respectively, allowing self-elimination of transgene-containing pollen and embryos. All the progeny from T_0 plants are transgene free and some of them are edited

demonstrating that the TKC system is very efficient in both editing the targets and removing the transgenes (He et al. 2018). The TKC system is easy to use and does not add more steps when constructing a TKC vector than the traditional CRISPR vectors. The specific gRNA-producing cassette is inserted into the TKC plasmid using conventional cloning techniques. The TKC gene-editing technology enables the isolation of transgene-free and target gene-edited plants within a single generation.

The first proof of concept TKC vectors used *CaMV35S* promoter to drive the *CMS2* expression. The *CaMV35S* promoter is a strong viral promoter that causes constitutive and ubiquitous gene expression. Although it was reported that CMS2 does not cause somatic cell death except pollen cells, the ubiquitous presence of CMS2 in plants is not ideal. Previous studies have shown that accumulation of CMS2 protein led to plants being more sensitive to drought than wild-type plants (Yu et al. 2015). Furthermore, it is known that the *CaMV35S* promoter does not work well in monocots compared to dicots (McElroy et al. 1990). The *CaMV35S* promoter in plants often undergoes epigenetic changes, leading to gene silencing (Weinhold et al. 2013). We replaced the *CaMV35S* promoter with two rice promoters: the rice *ACTIN1* promoter and the pollen-specific promoter *OsGEX2*, which is a strong and constitutive promoter. Because *CMS2* is a conserved pollen mitochondria-specific lethal peptide and BARNASE has been applied in many plants, it is very likely that the TKC system, which

relies on the suicide genes for self-elimination of transgenes, can be adopted for generating transgene-free and target gene-edited plants.

In summary, CRISPR-mediated gene-editing technology has successfully edited all major crops including rice, wheat, corn, cotton, soybean, and tomato. A huge amount of effort has been directed toward generating transgene-free and edited plants. It has been demonstrated that the CRISPR transgenes can be effectively eliminated by multiple approaches after target genes are edited. Marker-assisted selection of transgene-free plants and the TKC self-elimination of transgene technology are very effective in eliminating transgenes in plants that reproduce sexually. However, for non-sexually reproduced crops such as grapes and citrus, the RNP methods, nano-biotechnology, and transient expression of *CRISPR* genes are able to generate transgene-free and edited plants, but the efficiency is still low and intensive labor is needed. Further improvement of current technology and development of new technologies are still needed to effectively and effortlessly eliminate transgenes in both sexually and non-sexually reproduced crops.

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