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Review Article

Risk associated with off-target plant genome editing and methods for its limitation

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Assessment for potential adverse effects of plant genome editing logically focuses on the specific characteristics of the derived phenotype and its release environment. Genome-edited crops, depending on the editing objective, can be classified as either indistinguishable from crops developed through conventional plant breeding or as crops which are transgenic. Therefore, existing regulatory regimes and risk assessment procedures accommodate genome-edited crops. The ability for regulators and the public to accept a product focus in the evaluation of genome-edited crops will depend on research which clarifies the precision of the genome-editing process and evaluates unanticipated off-target edits from the process. Interpretation of genome-wide effects of genome editing should adhere to existing frameworks for comparative risk assessment where the nature and degree of effects are considered relative to a baseline of genome-wide mutations as found in crop varieties developed through conventional breeding methods. Research addressing current uncertainties regarding unintended changes from plant genome editing, and adopting procedures that clearly avoid the potential for gene drive initiation, will help to clarify anticipated public and regulatory questions regarding risk of crops derived through genome editing.

Introduction

Crops derived through genetic modification to express recombinant DNA [genetically modified (GM) crops] were first introduced for commercial use in 1996 and as of 2016 were grown on 185.1 million hectares in 26 countries [1]. Despite this widespread adoption and the lack of evidence for adverse effects to human health or the environment [2–5], GM crops continue to elicit concerns in many regions of the world and this has restricted their use to relatively few commodity crops and traits [6]. Because of the need for increased productivity, improved nutrition, and the wider use of crops as platforms for energy and value-added products, researchers worldwide are investigating new breeding technologies that may avoid the public controversy that hinders the widespread further use of GM crops [7].

Among new breeding technologies, genome editing has engendered particular interest as a way to rapidly and precisely create variation in crops while avoiding the presence of foreign DNA in the final product. This ostensibly reduces public concern and circumvents regulatory statutes that limit the entry of GM crops into the market [8]. The early promise for genome-edited crops to rapidly gain market entry has not proved to be the case. Europeans have avoided GM crops and are uncertain as to whether genome-edited crops should be subject to similar regulatory standards [9]. Initial regulatory opinion in the U.S.A. is that not all genome-edited crops are subject to regulation, but that sentiment may be shifting [10]. Here, we briefly describe the diversity of genome-editing tools and approaches that can be used for crop improvement and discuss the nature of concerns (both intrinsic and extrinsic) that may entail real or perceived risks of genome-edited crops.

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Diversity of tools and outcomes for genome editing

Genome editing uses engineered biomolecules (reagents) that can be directed to specific sites within the genome of a target organism to affect specific changes in DNA sequence through breaks and repairs or mismatches involving endogenous mechanisms. Repairs involving non-homologous end-joining (NHEJ) represent an error prone process where point mutations arise due to insertion or deletion of one or more nucleotides. Genome-editing reagents can also be used in conjunction with templates homologous to the break site to cause homology-directed repair (HDR). The templates inserted through HDR may range in size from short-nucleotide sequences to entire genes or multiple genes. Their source may be native to the organism or to a near relative, or they may represent foreign DNA which is synthesized or transgenic. The nature of these various genome-editing outcomes in crops has been the subject of extensive reviews [11–13].

The first demonstrated application of genome editing in plants involved the use of oligonucleotide-directed mutagenesis, where oligonucleotides bearing a desired nucleotide substitution but otherwise homologous to a specific genome site would associate as triplex DNA and incorporate mismatch edits using endogenous mechanisms [14]. Methodologies for genome editing with engineered nucleases (GEEN) have proved more robust and highly efficient. Numerous GEEN reagents–engineered meganucleases, zinc finger nucleases (ZFNs), transcriptional activator-like effector nucleases (TALENs) and clustered regularly interspaced short palindromic repeats and associated Cas9 endonuclease (CRISPR/Cas9)—have been developed and extensively reviewed for their efficiency, specificity, and application to various plant systems [15,16]. The emergence of the CRISPR/Cas9 reagent system with its relative ease of use, its modification by use of single-guide RNA (sgRNA) [17], and its improvement through redesign of the Cas9 nuclease [18] or adoption of alternative nuclease systems, such as Cpf1 [19], has led to the rapid and widespread use of genome editing as a preferred method to target highly specific changes to plant genomes.

The potential for adverse effects of off-target editing within the genome have been of intense interest to genome editing for human therapeutic applications where a key consideration is characterizing the occurrence of off-target edits on a genome-wide basis, since off-target edits could lead to adverse effects such as cytotoxicity, genotoxicity, or potential chromosomal rearrangements [20]. Unlike therapeutic applications in the cellular and mammalian system, where these potential adverse effects have direct implications to the treatment outcome, any toxic effects in plants are largely a technical challenge for accomplishing the genome edit. This is because the subsequent process of plant regeneration and trait segregation through breeding selection to obtain a desired plant genome edit enables the detection and evaluation of off-target edits to restrict unwanted downstream phenotypic effects. Furthermore, the combined abilities for stress resistance and accurate DNA repair mechanisms in plants contribute to the genetic stability of the resulting edit in crops. Therefore, concerns associated with editing in cellular and mammalian systems are not directly applicable to crops [10]. These concerns must be borne in mind, however, because they raise questions regarding safety from both regulators and the public.

Risks directly associated with off-target edits to crop genomes

The development of genome-editing reagents has progressively focused on ZFNs, TALENs, and the CRISPR/Cas9 system. The choice of system relates to ease and breadth of use and the efficiency and specificity of editing. Both ZFNs and TALENs function as dimers with each monomer comprising a fusion protein-binding domain which together determine sequence specificity; cleavage is carried out by the attached FokI nuclease catalytic domain [13,21,22]. Successful applications of ZFNs and TALENs have been shown in mammalian cells, plant cells, and other organisms [17,23–27]. Unfortunately, because of low affinity and low specificity, gene editing with ZFNs has displayed high frequencies of off-target edits and high toxicity [13]. The design of TALENs involves one-to-one recognition rules between protein repeats and nucleotide sequences, which results in improved editing efficiency and reduced off-target effects [21,28]. It is difficult, however, to construct the nuclease protein and a new TALEN protein must be generated for each DNA target site, which increases time and costs for development [29]. Additionally, the cut efficiency for TALENs varies depending on target sequence and TALENs cannot target methylated DNA [13]. As we know, DNA methylation of plant genome controls plant growth, development, transcription, DNA repair, gene expression, and cell differentiation, and is particularly involved in specific gene silencing. Moreover, the levels of DNA methylation from resistance-related genes are related to environmental stresses such as chilling, salt, heavy-metal, or pathogenic infection. Both

DNA methylation and hypermethylation contribute to the adaptation of plants to stress and stable inheritance [30–32], and thus, TALENs have limited application for these types of breeding goals. Compared with ZFNs and TALENs, the CRISPR/Cas9 system exhibits greater simplicity, efficiency, and versatility, and it also can target methylated DNA [13,33]. CRISPR/Cas9 has been widely adopted for genome editing in plants. Over 25 plant species and over 100 genes have been subject to successful editing with CRISPR/Cas9, and many desirable traits in major crops have been created [13,34–36]. However, a crucial current concern for the CRISPR/Cas9 system is the potential for higher off-target effects than with TALENs [21,29,37], which must be addressed for widespread use in crop breeding and gene therapy [38,39].

Beyond reagent toxicity that may limit the ability to efficiently genome edit in some systems, there are no risks inherent to the genome-editing process *per se*; rather some risks associated with downstream attributes of the phenotype derived from genome editing may be ascribed to unintended changes in the genome [40]. Therefore, genome-wide characterization for off-target edits serves as an indicator of potential undesired phenotypic changes in genome-edited crops. In order that there is integrity in the use of comparative assessments to evaluate off-target effects in genome-edited crops, research must focus on how to alleviate off-target edits and how to assess the frequency and nature of those off-target edits that do occur [41].

Early work with CRISPR/Cas9 reported that off-target mutations in human cancer cells were up to 50% more common than mutations at the on-target sites, which were due to dysfunctional DNA repair mechanisms and promiscuous sgRNA [21,37,42]. The specificity of the CRISPR/Cas9 system mainly depends on the sgRNA seed sequence within 10–12 bp directly 5′ of the NGG protospacer-adjacent motif (PAM-proximal) [37]. When the sgRNA sequence recognizes partial mismatches outside the seed sequence instead of on-target sites, then off-target edits will be produced. With the further development of CRISPR/Cas9 techniques benefiting from greater specificity in sgRNA design, minimal off-target binding activity and the absence of off-target mutations have been reported in zebrafish [43], mice [44], chicken [45], stem cells [46], *Arabidopsis* [47], and rice [48]. Progress in targeting has led to guidelines for minimizing CRISPR/Cas9 off-target effects [49].

When considering the incidence of off-target edits that can occur from crop genome editing, it is important to recognize the comparative source and magnitude of genome alterations that occur in plants under natural conditions. Regardless of source, mutation in higher plants can result in error-prone repairs some of which may induce direct toxic effects to reduce protein synthesis, destruct cell membrane and photosynthetic protein to inhibit plant growth, lead to chromosome fusions, or produce genetic changes in plant populations that may be passed on to the next generations [50,51]. Plants are constantly exposed to environmental stresses, including UV-B radiation, ozone, desiccation and rehydration, and air and soil pollution, which can cause a range of DNA damage products including single-strand (SSBs) and double-strand DNA breaks (DSBs) due to stress response [50,52,53]. The DSBs are considered one of the most serious forms of DNA damage, since when DSBs cannot be repaired precisely, they may lead to cell death, loss of genetic information, or genotoxic effects [54]. In higher plants, DSBs are mainly repaired by NHEJ mechanisms [50,55]. To maintain genome stability, fertility, and genetic diversity, there must be accurate DNA repair mechanisms in coding regions, and error-prone repair serves as a source of natural mutational variation important for plant evolution and useful for crop improvement [52,53,56]. Plant breeders have traditionally generated plant mutations using chemical or physical genotoxins. In this case, DSBs are induced randomly in the genome, and error-prone repair very rarely generates desirable phenotypes [57]. Over 3000 crop varieties have been produced using radiation mutagenesis and are used worldwide without regulatory risk assessment (with the exception of Canada) [58].

The DSBs in coding regions introduced by CRISPR/Cas9 or other genome-editing reagents will also depend on plant endogenous repair pathways that may insert or delete fragments to induce mutation. Thus, genome modifications by CRISPR/Cas9 or other genome-editing reagents are much more site-specific than similar modifications sought by traditional plant breeding approaches, and those DSBs occurring in off-target sites probably are comparable to naturally induced mutation [57]. The recent suggestion that genome-wide off-target effects from targeted CRISPR/Cas9 editing in mammalian cells are much more prevalent than previously believed and are not associated with near-homologous targets [39] is likely attributable to pre-existing variants in the cell lines studied [59], but the attention this result has garnered heightens the need for unbiased genome-wide characterization of CRISPR/Cas9 editing in order to understand and guide crop development based on genome-editing technology.

In terms of the actual mechanism of targeting CRISPR/Cas9 through the design of the sgRNA, the CRISPR/Cas9 system has similarities to RNA interference (RNAi), and what is more, in some circumstances, RNAi can also trigger nonspecific effects or off-target effects [60]. Early studies off-target effects of RNAi focused on

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strong overall complementarity with target DNA, when in fact it is the 3′-untranslated region short seed segment of the antisense strand of the small interfering RNA that allows for off-target effects; recognition of this led to improved design and targeting strategies for RNAi [61]. Fortunately, lessons learned from the RNAi field have caused researchers to pay more attention to the seed region of CRISPR sgRNA quite early in their studies, and this has revealed some sequence flexibility and tolerance for mismatches and bulges, which suggested approaches on how to increase the specificity and decrease the sequence-directed off-target cleavage [62–64]. Additionally, in RNAi systems, when short-hairpin RNA expression outcompetes that of endogenous microRNAs, it will lead to the breakdown of cellular regulation [65]. This is similar to the effect of overabundance of sgRNA-Cas 9 complexes to affect off-target efficiency [29,66,67]. Finally, the delivery vector used in the RNAi system such as cationic lipids with the high concentrations can also trigger unwanted responses [68], and these unwanted effects exist for CRISPR/Cas9 as well [60].

Methods to minimize off-target effects of plant genome editing

The nature of PAM recognition is proving to be more complex than the earlier understanding of the CRIPSR/Cas9 system would indicate; and potential off-target sites upstream of the 5′-NAG PAM, 5′-NGG PAM, 5′-NGA PAM, and perhaps other PAM types should be considered when designing a CRISPR reagent [37,48,69]. What is more one or two mismatches within the seed sequence of the sgRNA in the PAM proximal region will also induce higher numbers of off-target edits [37], so many sgRNA design tools support the identification of specific sgRNA sequences to improve targeting and minimize off-target effects. Among CRISPR design tools, CGAT, Crispr-P, CHOPCHOP, and CRISPR contain all the functionalities necessary to predict and rank potential off-target locations within plant genomes [70]. Despite the availability and increasing quality of these design tools, there remain further methodological questions of how to optimize the ratio of on-target to off-target edits and of how to control the dosage of the transfected DNA and the level of the Cas9–sgRNA complex.

A further option for improving target specificity and concomitantly reducing the incidence of off-target edits is to utilize CRISPR nickase systems. For instance, a catalytic mutant of *Streptococcus pyogenes* Cas9 (SpCas9) can be used as a nickase to induce SSBs or as a paired nickase system to cause offset DSBs. The use of this system resulted in highly specific gene editing without detectable damage at known off-target sites [37,71,72]. The use of a nickase system is reported to substantially reduce the mutation frequency without affecting the HR repair in *Arabidopsis thaliana* compared with wild-type Cas9 nuclease codon optimized for *A. thaliana* [73]; and when using paired nickases for editing cell lines, off-target activity was reduced even more appreciably when compared with wild-type Cas9 [74].

The use of truncated sgRNA of 17 or 18 nt with a shortened 5′-end greatly decreased undesired mutagenic effects at off-target sites in mammalian cell systems without sacrificing on-target genome-editing efficiencies [63]. Similarly, in plants, use of truncated sgRNA in a CRISPR/Cas9 system using a constitutive promoter resulted in high on-target mutation rates with no off-target effects detected [75]. Moreover, combining truncated sgRNAs with pairs of Cas9 nickase led to further reductions in off-target mutations [63]. When researchers developed the fusion of catalytically inactive Cas9 (dCas9) to FokI nuclease with sgRNA sequence to form the RNA-guided fCas9, they found DNA cleavage by fCas9 required association of two fCas9 monomers that simultaneously bind target sites ∼15 or 25 bp apart. In human cells, researchers showed fCas9-modified target DNA sites with >140-fold higher specificity than wild-type Cas9 and at least 4-fold higher specificity than that of paired nickases at loci with highly similar off-target sites [76,77].

In addition to these strategies for directly reducing unwanted off-target mutations, other research advances and technological strategies can act to decrease the possibility of unintended effects from genome editing of crops by improving the efficiency of editing to the target site while reducing opportunities for unintended off-target mutations. For instance, concentration–time (dose-dependent) effects of the reagent may alter ratios of on-target and off-target edits prior to breeding selection to eliminate the gene-editing reagent [78]. In addition, the delivery of preassembled Cas9 protein-gRNA ribonucleoproteins (RNPs) (that is a DNA-free method) instead of plasmids can control Cas9 protein concentration to limit genotoxicity, can alleviate overexpression of sgRNA to reduce off-target effects, and can avoid transfer of genome-editing reagents to the next generation [79,80]. The possibility for RNP delivery to plant cells has been shown with biolistic particle bombardment where delivery of recombinase led to site-specific, heritable edits [81]. In higher plants, the traditional

Agrobacterium-mediated plasmid delivery system not only can guarantee success for delivering of genome-editing reagents, but may also control transgene copy numbers to moderate concentration of sgRNA : Cas9 complexes and thus, reduce off-target activity [82]. Moderate culture conditions such as acetosyringone in the culture medium can induce or suppress the expression of some non-coding RNA from *Agrobacterium* to further influence gene-editing efficiency [83]. And selectable markers like phosphite oxidoreductase may prove effective to screen and recover more transformants to improve efficiencies for on-target editing [84].

Finally, numerous advances continue to improve the ability to conduct comprehensive genome-wide characterization for the detection of off-target sites. Sanger and deep sequencing are increasingly used to analyze the off-target effects of CRISPR/Cas9 edits based on library construction of on-target and potential off-target regions and the evaluation of the exome-captured library [62]. Chromatin immunoprecipitation and high-throughput genome sequencing (ChIP-seq) has been used to reveal a well-defined seed region for target binding and to identify a very large number of off-target binding sites in cellular systems [85–87]. Efficient detection of off-target sites in human cells was shown with GUIDE-seq (genome-wide, unbiased identification of DSBs enabled by sequencing), which can detect novel off-target sites through the capture of double-stranded oligodeoxynucleotides into DSBs [77]. The method Digenome-seq relies on whole-genome sequencing of cell-free genomic DNA digested *in vitro* using a nuclease of interest [42]. When used in a multiplex configuration (Digenome-seq) revealed *bona fide* off-target sites that had been missed by GUIDE-seq [49]. Despite these advances, researchers continue to explore various techniques to detect the off-target sites that may be predictive of unintended risks. Most of the abovementioned strategies such as ChIP-seq, GUIDE-seq, and Digenome-seq have been successfully used to detect off-target sites in human cells but not in plants. Researchers need to establish more approaches and databases appropriate to plant systems in order to gain confidence as to the characterization of off-target editing frequencies in genome-edited crops.

Potential for unintended gene drive generation

In addition to consideration of off-target edits, researchers need to consider the ecological implications of unanticipated downstream effects when genome editing is used for plant improvement or for control of weeds/invasive plants, and when inadvertent gene drive generation occurs because encoded CRISPRs are not segregated from edited plants [10]. Although gene drives occur in nature [88], if inadvertently or indiscriminately deployed as a global gene dive, for instance as an invasive species control measure, the CRISPR/Cas9 system may promote rapid, unintended gene propagation within a species. Unintended release of plants containing global gene drives from confinement under conditions where there is outcrossing, a relatively short generation time, the stability of the driving genetic elements and suitable population structure could allow entry into wild populations to introduce undesired phenotypes and perhaps affect plant diversity [89,90]. Such an outcome is highly unlikely in conventional plant breeding systems, but there remains the possibility for local gene drive dissemination when the DNA encoding an engineered nuclease is not removed and may be linked to the editing site so as not to readily segregate. Broad recognition of this potential by the research community has led to alternative design strategies such as daisy chain drives as well as research governance approaches intended to recognize and forestall unintended gene drive release [10]. Research has shown that the potential for drive systems capable of overwriting other gene drives is a means to correct and minimize unwanted genome editing in wild yeast strains (*Saccharomyces cerevisiae*) [91], suggesting that there are technological options to mitigate gene drive risks. More deliberation as to gene drive use will be needed for both research and governance [92]. In a word, gene drives and CRISPR technology must be controlled by design, observation, segregation, and an appropriate mix of governance mechanisms [10,93].

Downstream risks of genome editing for crop improvement

In terms of process-based risks, further improvements in targeting, off-target molecular characterization and adoption of methodologies to avoid or characterize the presence of foreign DNA in plant germplasm advanced into crop breeding programs will minimize the opportunity for undesired outcomes of the genome-editing process itself. Further characterization for downstream risks associated with a specific genome-edited crop should be conducted as appropriate for the nature of the phenotype generated and its proposed uses [10]. For instance, for the purposes of food safety assessment, genome-edited crops can logically fall into two classes. If the crop contains no foreign DNA because of the nature of editing reagents used (e.g. an RNP and introduced sgRNA) or because post-editing segregation removes foreign DNA elements, the crop cannot be differentiated from crops derived using traditional plant breeding methods (including chemical or radiation mutagenesis).

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This class of genome-edited crop should be subject to the same types of regulatory consideration as are traditionally bred crops. On the other hand, if there is evidence for foreign DNA insertion without subsequent removal, the genome-edited crop is indistinguishable from a GM crop and therefore should be given the regulatory consideration as appropriate for a GMO. In GM crop risk assessment, regulators determine substantial equivalence to enable safety assessments for the GM crop. This entails principally compositional analysis in addition to molecular characterization to determine nutrient, anti-nutrient, and toxicant presence and concentration when compared with non-GM conventional counterpart crops [94].

If potential downstream risks cannot be avoided, hazard identification and problem formulation are needed to appropriately direct the subsequent the risk assessment process. In the future, a further aspect of downstream risk assessment may be in the use of 'omics' analyses (transcriptomics, proteomics, metabolomics epigenomics, and miRNomics) to study dynamic changes of GM crops [95–97]. While these methodologies may see increasing future use and application toward genome-edited crops, they, at present, have limited regulatory applicability as the outcomes of 'omics' analyses are not well defined in the context of regulatory decision-making and the paradigm for comparative risk assessment [10].

Balancing intrinsic and extrinsic concerns regarding crop genome editing

The foregoing discussion has focused on consideration of extrinsic risk; that is, factors regarding the development and use of genome-edited crops that can be evaluated on the basis of empirical information describing the potential for adverse outcomes when a genome-edited crop is used as food or is released to the environment. Some elements of the public, however, may have concern regarding genome-edited crops that reflect intrinsic aspects of the process of genome editing of a crop in and of itself. These are less readily addressed since they relate to perceptions of risk arising from beliefs and may involve elements of tradition and culture that are ultimately intractable from a science perspective. The tension between intrinsic and extrinsic elements of risk for genome-edited crops is found in arguments regarding whether the process of genome editing in and of itself should be the focus of regulatory considerations or whether the risk associated with the derived products is a more relevant consideration [98]. Differing regulatory domains throughout the world demonstrate the difficulties in balancing these concerns. For instance, countries which have been able to successfully deploy GM crops have tended to follow a path more focused on measurable risk (extrinsic concerns); while in the EU, debate is ongoing as to whether regulation should focus on the process itself and thus require comprehensive assessment even when there is no evidence a gene-edited crop differs from conventionally bred counterparts (a manifestation of intrinsic concern) [99].

In the human germline engineering field, the use of the CRISPR/Cas9 system faces clear ethical issues because of unanticipated effects of editing [100], and this concern will bleed over to considerations of crop genome editing. Clearly, more research and continued public discourse and deliberation about gene-editing technology and concerns from various perspectives will be needed for both human therapeutic use and crop improvement. For the research community, however, the immediate path forward must address uncertainties regarding off-target editing and unintended effects which will help to clarify regulatory approaches and safety assessment procedures [101]. Current technology can only alleviate off-target effects; it can neither eliminate off-targets nor can it clearly answer the extent that this should constitute a downstream concern. Moreover, updated methods are finding more novel off-target sites in human cells than by classical technology [38,39]. In plants, there is limited evidence for common occurrence of off-target sites for various reasons including the characteristics of plant genomes, their environment, or limitations in research information. The history of crop breeding to date illustrates the use of variation that is the outcome of natural or induced random mutagenesis, and this is widely accepted [101]. However, the information bridging this knowledge to genome editing is limited to experts in the field. We suggest that regulators and policy planners need to support research on detection/prediction of off-target sites and unintended effects to help establish a safety assessment paradigm based on molecular characterization and phenotypic attributes of the derived products. Scientists and developers need to continue to advance and communicate product concepts based on benefits to consumers and to widely publish and communicate progress in gene editing of crops to ensure the transmission of information to improve consumer understanding and to encourage public discussion.

Summary

- Plant genome editing represents a wide variety of potential reagents and methodologies with potential outcomes for which off-target effects may be consequential.
- Robust methods for analysis and interpretation of genome-wide frequencies of off-target effects are useful indicators of potential inadvertent outcomes in the downstream product of genome editing.
- Comparative risk assessments that do not show frequencies of off-target effects of genome editing above baseline effects from traditional methods of plant breeding indicate a low probability for adverse outcomes of the genome-editing process *per se*.
- Once assurances of process integrity are established for a given genome-editing process, the evaluation of the derived product of genome editing (the desired phenotype) should be the focus of risk considerations.

Abbreviations

ChIP-seq, chromatin immunoprecipitation and high-throughput genome sequencing; CRISPR/Cas9, clustered regularly interspaced short palindromic repeats and associated Cas9 endonuclease; DSBs, double-strand DNA breaks; GEEN, genome editing with engineered nucleases; GUIDE-seq, genome-wide, unbiased identification of DSBs enabled by sequencing; HDR, homology-directed repair; NHEJ, non-homologous end-joining; PAM, protospacer-adjacent motif; RNAi, RNA interference; RNPs, ribonucleoproteins; sgRNA, single-guide RNA; SpCas9, *Streptococcus pyogenes* Cas9; SSBs, single-strand breaks; TALENs, transcriptional activator-like effector nucleases; ZFNs, zinc finger nucleases.

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Competing Interests

The Authors declare that there are no competing interests associated with the manuscript.

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