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ORIGINAL RESEARCH

Achieving Plant CRISPR Targeting that Limits Off-Target Effects

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ABSTRACT

The CRISPR-Cas9 system (clustered regularly interspaced short palindromic repeats with associated Cas9 protein) has been used to generate targeted changes for direct modification of endogenous genes in an increasing number of plant species; but development of plant genome editing has not yet fully considered potential off-target mismatches that may lead to unintended changes within the genome. Assessing the specificity of CRISPR-Cas9 for increasing editing efficiency as well as the potential for unanticipated downstream effects from off-target mutations is an important regulatory consideration for agricultural applications. Increasing genome-editing specificity entails developing improved design methods that better predict the prevalence of off-target mutations as a function of genome composition and design of the engineered ribonucleoprotein (RNP). Early results from CRISPR-Cas9 genome editing in plant systems indicate that the incidence of off-target mutation frequencies is quite low; however, by analyzing CRISPR-edited plant lines and improving both computational tools and reagent design, it may be possible to further decrease unanticipated effects at potential mismatch sites within the genome. This will provide assurance that CRISPR-Cas9 reagents can be designed and targeted with a high degree of specificity. Improved and experimentally validated design tools for discriminating target and potential off-target positions that incorporate consideration of the designed nuclease fidelity and selectivity will help to increase confidence for regulatory decision making for genome-edited plants.

Core Ideas

- Plant CRISPR-Cas9 genome editing may generate unintended off-target mutation.
- Potential for off-target mutation is an important regulatory question for genome-edited plants.
- Validated design approaches to discriminate target and potential off-target edits are needed.

THE CRISPR-Cas9 system has rapidly emerged as the preferred method for genome editing with engineered nucleases (GEEN). A CRISPR system can easily and inexpensively direct modification of endogenous genes in a wide variety of cell types and organisms (Sander and Joung, 2014). However, the development of plant genome editing to date has tended to emphasize efficacy and efficiency of generating a desired targeted change with lesser consideration of the potential for off-target mismatches that may lead to unintended changes within the genome. Considerations in human therapeutic applications of GEEN provide insight as to potential effects in plant systems that may improve effectiveness of the editing process and may inform the needs for biotechnology

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Abbreviations: CRISPR, clustered regularly interspaced short palindromic repeats; DSB, double-strand break; GEEN, genome editing with engineered nucleases; gRNA, guide RNA; nt, nucleotide; PAM, protospacer adjacent motif; RNP, ribonucleoprotein; SNV, single-nucleotide variation; SpCas9, *Streptococcus pyogenes* Cas9; TDS, targeted deep sequencing; WGS, whole-genome sequencing.

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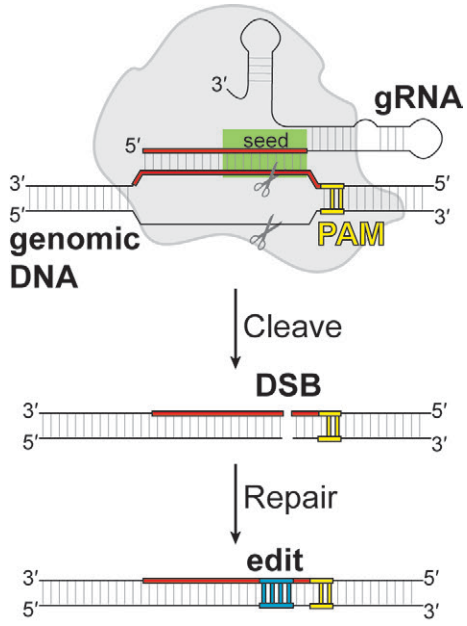

Fig. 1. Binding, cleavage, and repair using the CRISPR-Cas9 system. The Cas9 protein from *Streptococcus pyogenes* (SpyCas9) and associated guide RNA (gRNA), containing a 20-nt recognition sequence, will cleave a target sequence located upstream of the protospacer adjacent motif (PAM) region. The SpyCas9-associated gRNA is highly specific for the 10- to 12-nucleotide seed sequence immediately upstream of the PAM sequence.

product safety assessments. Current research consensus (Corrigan-Curay et al., 2015) regarding identifying and evaluating the impact of off-target activity for human therapeutic applications emphasizes unbiased analyses of genome breaks (analytical strategies for characterizing genome-wide specificity) that could have downstream effects such as cytotoxicity, genotoxicity, and potential chromosomal rearrangements. For plant applications, unbiased analyses will be useful for understanding the specificity for increased editing efficiency as well as for assessing the likelihood for unanticipated downstream effects from off-target mutations. Increasing specificity for targets while decreasing the chances of off-target changes entails the development of improved design methods. These include better prediction of the prevalence of off-target mutations as a function of genome composition and design of the engineered RNP. Greater assurances as to the downstream effects of genome editing will be useful for increased scientific, regulatory, and public acceptance of the technology.

Background

During genome editing, CRISPR-associated Cas9 is directed by a guide RNA (gRNA) to a complementary sequence within the target region (Fig. 1). Following target binding, Cas9 cleaves the DNA (Jinek et al., 2012), creating a double-strand break (DSB) that introduces mutations on DNA repair (Doudna and Charpentier, 2014). Although the mechanism of Cas9 target binding and cleavage has been elucidated (Jinek et al., 2012; Sternberg et al., 2014, 2015), the rules for targeting are less well understood (Doench et al., 2014). Theoretically, the only limitation for gRNA design is the presence of a protospacer adjacent motif (PAM), a short motif required for Cas9-target binding (Jinek et al., 2012). However, early studies clearly indicate that gRNA design will greatly influence the specificity of Cas9 (Fu et al., 2013; Hsu et al., 2013). Genome-wide studies of binding specificity indicate that there may be high levels of transient Cas9 binding without subsequent generation of DSBs (Kuscu et al., 2014; Wu et al., 2014). The observation of more promiscuous binding than cleavage for Cas9 reflects the dependency of Cas9 cleavage on both the PAM sequence and gRNA complementarity to the target sequence (Sternberg et al., 2015).

Whole-genome sequencing (WGS) for frequency of mutations induced by DSBs is complicated by comparisons among test and reference genomes, the presence of single-nucleotide variations (SNVs), and the inability for limited sequencing depth to detect low-level mutations (O'Geen et al., 2015). These issues, along with procedural variance in genome assembly techniques for families of small size reads and the inability to effectively deal with repetitive regions, have led regulators to defer use of WGS for the present and to rely on more conventional molecular characterization techniques for purposes of risk assessment (Pauwels et al., 2015). Deep sequencing at putative off-target sites provides direct validation of off-target mutation frequencies as predicted in silico, but uncertainty remains regarding unanticipated genome-wide effects that may be missed. Improved computational tools and algorithms will help to decrease the uncertainty for such unanticipated mutations (O'Geen et al., 2015).

Bioinformatics tools are used in the design of gRNAs to identify both target and off-target sites, since genome editing using the CRISPR-Cas9 system may result in off-target effects that can complicate research and impact regulatory assessment (Brazelton et al., 2016). These tools select gene regions for gRNA design based on specific sequence characteristics such as size (usually 21–23 bases) and specific nucleotide constitution. For any given application, follow-on studies are necessary to ascertain the degree of off-target mismatches that have ultimately occurred in the genome editing process (O'Geen et al., 2015). Currently, bioinformatics predictions lack validation as to target specificity for understanding whether the predictions are in fact real. Experimental data that consider the attributes of the design tool along with design criteria for the gRNA can lead to improved algorithms for more reliable prediction of off-target genotypic effects for genome-edited plants. Advances in this direction have occurred for human therapeutic applications (Corrigan-Curay et al., 2015) but are largely lacking for the design of plant gRNAs. Differences are anticipated for plant applications because of widespread large-scale genomic duplication (including not only segmental but also ploidy-level genomic duplication).

The preponderance of scientific evidence indicates enhanced genetic instability from transgenesis is unlikely

in plants (Weber et al., 2012; Schnell et al., 2015). Regardless, the use and acceptance of CRISPR-Cas9 or other genome editing approaches requires specific analysis to improve fundamental understanding of editing efficiencies and to answer questions regarding downstream effects of off-target mutations. Off-target mutation frequencies resulting from genome editing must be addressed in comparison with baselines reflecting both natural and chemical- or irradiation-induced mutation. This comparison requires highly sensitive determinations on a genome-wide basis. For instance, the spontaneous mutation rates in the nuclear genomes of *Arabidopsis thaliana* (L.) Heynh. were determined as 7×10^{-9} base substitutions per site per generation representing predominately base substitutions and, to a lesser extent, insertions or deletions (Ossowski et al., 2010). The difficulty in making this comparison through genome-wide analysis was shown in soybean [*Glycine max* (L.) Merr.], where structural variations in transgenic plants were one order of magnitude less than for radiation-induced mutants and two orders of magnitude less than observed between cultivars (Anderson et al., 2016).

Off-Target Effect Analysis

Typical approaches to gauging mismatch potential for a given CRISPR-Cas9 include WGS, targeted deep sequencing (TDS), and Cas9 binding assays (O'Geen et al., 2015). There are procedural limitations to these methods for robust assessment of off-target mutations attributable to any given CRISPR-Cas9 application. Whole-genome sequencing should hypothetically identify mutations occurring throughout an edited genome but will be limited as a result of incomplete sequence coverage and alignments. The use of TDS, which evaluates off-target positions within genomes based on homology to the target site, may be preferable to WGS for evaluation of frequencies of off-target positions. This method needs to be used in conjunction with robust bioinformatics tools for identification of near homologous sites and will not assess off-target sites that are not predicted if there are discrepancies between the reference genome and the genome being analyzed (see Improving Genome Sequence Quality and the Bioinformatics Tools that Make Predictions section).

The quest for better understanding of off-target effects has led to unique approaches to detection of off-targets resulting from CRISPR RNA-guided nucleases which as of yet have not been applied to plant systems. Genome-wide unbiased identification of DSBs enabled by sequencing uses capture of double-stranded oligodeoxynucleotides for global detection of DNA DSBs introduced by engineered nucleases (Tsai et al., 2015). High-throughput, genome-wide translocation sequencing is a versatile assay for detecting nuclease off-target sites that may also reveal collateral recurrent translocation is it occurs between target and off-target DSBs (Frock et al., 2015). Sequencing in vitro nuclease-digested genomes (digenomes) yielded sequence reads with the 5′ ends at predicted off-target cleavage sites that validated off-target sites where insertions or deletions were induced with frequencies near the detection limit of targeted deep sequencing (below 0.1%) (Kim et al., 2015).

The failure to appropriately account for duplicate genes and to exclude intragenic regions further complicates interpretations of off-target mutations. Appropriate controls and reference genomes are critical to appropriately discriminate off-target mutations arising from the CRISPR-Cas9 reagent, since some instance of baseline mutation will be attributable to natural processes of spontaneous mutation (Ossowski et al., 2010), in vitro cell culture systems (see Impact of In Vitro Cell Culture on the Genome Editing Process section), and false positive error reporting.

Cas9 gRNAs can accommodate mismatches varying by as many as five nucleotides in the 5′ upstream region, but they are highly specific for the seed region representing the 10 to 12 nucleotides (nt) immediately upstream of the PAM sequence (Cho et al., 2014; Hsu et al., 2013; Pattanayak et al., 2013). Thus, there appears to be ample opportunity for multiple sites of off-target DNA cleavage in genomes of even moderate size (Pattanayak et al., 2013). Selecting unique sequences differing by as few as two bases can reduce off-target effects to levels below the sensitivity for analysis by deep sequencing (mutation frequencies of 0.1 to 0.01%) (Cho et al., 2014).

Using both WGS and TDS, Yang et al. (2014) did not detect gross genome alterations or elevated mutation rates from CRISPR-Cas9 cellular editing in human cell lines. They did, however, find that a SNV that reduced an off-target sequence from three to two mismatches in the region immediately upstream of the PAM sequence allowed for increased off-target cleavage by the nuclease. Given that such SNVs may occur with relatively high frequency within portions of some genomes, their significance for mutations of functional consequence warrants consideration. In addition to specificity imparted through gRNA composition, off-target edits have been shown to decrease with reduced tail length of the gRNA complex, but this came at the expense of lowered efficiency of target edits (Hsu et al., 2013; Pattanayak et al., 2013).

Compared with more extensive information in mammalian systems (Corrigan-Curay et al., 2015), data describing the off-target effects of CRISPR-Cas9 editing in plants are currently limited. To date, there has been little effort to determine whether gRNA or Cas9 modification can affect specificity for plant genome editing other than to use species-specific codon optimization for the Cas9 gene (Bortesi and Fischer, 2015). Additionally, off-target effects in plant systems have not benefited from rational design approaches to quantify the off-target frequencies and to clarify the underlying factors that may govern target specificity. The data that are available, however, suggest infrequent off-target mutations from CRISPR-Cas9 genome editing in plants (Table 1).

Table 1. Plant CRISPR studies where off-target mutations were analyzed.

Species	Cas9 codon optimization	Cas9 promoter	gRNA promoter	Off-target effect	Generation monitored†	Off-target analysis‡	Reference
Transient transfection							
Arabidopsis thaliana	Arabidopsis	35SPPDK	AtU6	No	NA	No mutation was detected in a near-homolog to the target containing two mismatches to the 12-bp seed sequence	Li et al., 2013
Citrus sinensis	Human	CaMV35S	CaMV35S	No	NA	No mutation for eight sites with 13- to 17-bp out of 20-bp identity to the target sequence	Jia and Wang, 2014
Nicotiana benthamiana	Eukaryotic	CaMV35S	AtU6	No	NA	No mutation for 18 sites with 14- to 17-bp out of 20-bp identity to the target sequence	Nekrasov et al., 2013
Oryza sativa	Rice	2xCaMV35S	OsU3	Yes	NA	Mutations occurring at one of three near-homologous sites with 1- or 3-bp mismatches to the target sequences	Shan et al., 2013
Oryza sativa	Human	CaMV35S	OsU3 or OsU6	Yes	NA	Mutations occurred at one of three mismatch sites containing one mismatch in the 15-bp seed sequence; mutation occurred for a mismatch at the 11 position proximal to the PAM but not at the 1 or 9 position	Xie and Yang, 2013
Stable transformation							
Arabodopsis thaliana	Human	CaMV35S	AtU6	No	T1, T2	Whole-genome sequencing of T1 and T2 plants at a sequencing depth of ~60×	Feng et al., 2014
Glycine max	Human	CaMV35S	MtU6	Yes	T0	Three of 15 potential off-target sites with two to six mismatches were edited in a range of frequencies	Jacobs et al., 2015
Nicotiana tabacum	Tobacco	2xCaMV35S	AtU6	No	T0	No mutation for one potential off-target site with a perfect match to the 12-bp seed sequence and having 18-bp out of 20-bp identity with the target sequence	Gao et al., 2015
Oryza sativa	Rice	2xCaMV35S	ZmU3	No	T0	Whole-genome sequencing of seven T0 plants	Zhang et al., 2014
Oryza sativa	Rice	2xCaMV35S	ZmU3	Yes	T0	One mutation in analysis of 13 putative mismatch loci containing from one to six mismatched bases; the susceptible loci had a 1-bp mismatch outside the seed region of the target site and showed mutation for seven of 72 T0 plants	Zhang et al., 2014
Oryza sativa	Plant	2xCaMV35S	AtU6	No	T0	No mutation detected for three near-homologous sites with 1-base or 3-base mismatches to the target sequence	Xu et al., 2014
Oryza sativa	Rice	ZmUbi	OsU6	No	T0	No mutations occurred at six sites that contained 16 or more nucleotide matching the target sequence	Zhou et al., 2014
Oryza sativa	Rice	2xCaMV35S	OsU3	Yes	T0	Frequent mutations of a homologous sequence with a 1-bp mismatch 17-bp 5′ upstream of the PAM sequence	Endo et al., 2014
Populus trichocarpa	Human	CaMv35S	MtU6	No	T0	No mutation in closest homolog with four mismatches including one in PAM; no mutation in transgenic	Zhou et al., 2014
Zea maize	Maize	ZmUbi	ZmU6	No	T0, T1	No mutations for eight sites with five or fewer mismatches to the target sequence	Zhu et al., 2016

† NA, not applicable.

‡ PAM, protospacer adjacent motif.

Improving Genome Sequence Quality and the Bioinformatics Tools that Make Predictions

The specificity of *Streptococcus pyogenes* Cas9 (SpCas9) for editing a particular gene can be maximized by identifying and avoiding potential off-target genomic sequences using bioinformatics tools. Computational tools inform the selection and validation of single-guide RNAs to minimize mismatches to off-target loci for genome editing applications by incorporating algorithms that account for the degree that SpCas9 tolerates mismatches between guide RNA and target DNA at different positions in a sequence-dependent manner; the sensitivity to the number, position and distribution of mismatches; or the optimization of concentration of SpCas9 and single-guide RNA to favor target vs. nontarget cleavage (Hsu et al., 2013). There are a number of bioinformatics tools currently available to guide CRISPR-Cas9 editing of plant genomes (Brazelton et al., 2016), but validation of their predictability for limiting nontarget effects is largely lacking.

One challenge in using bioinformatics tools for discerning off-target effects of genome editing is that plant reference genomes may be limiting. Only a few lines or cultivars may be well sequenced for any given plant species, and many crop plants lack an assembled genome entirely (Shangguan et al., 2013). In addition, no current plant reference genome sequence is complete except in draft quality; regions of the genome are missing or poorly assembled (Tello-Ruiz et al., 2016). For those species that do have a reference-quality genome, some duplicate genes and gene families are anticipated to be poorly represented in the assembly, making it difficult to assess all possible changes that could be induced by a given CRISPR construct. To reduce the potential impact of this problem, improved reference genome sequences and additional genome sequences for diverse germplasm are sorely needed. Once these resources exist more generally, experiments to assess the performance of existing CRISPR design algorithms for both target and off-target changes can be assessed empirically, and changes to methodologies for reduced off-target effects can be implemented.

Improving Cas9

Improvements to the specificity as well as efficiency and fidelity of the Cas9 enzyme are anticipated to reduce off-target activity. Pursuit of these sorts of improvements requires multiple, simultaneous approaches. For instance, off-target edits may be minimized by lowering the cellular concentrations of GEEN reagents (Hsu et al., 2013; Pattanayak et al., 2013), since dose-dependent reagent effects (reagent concentration over time) represent an opportunity for increased target and off-target edits prior to null segregant breeding selection to eliminate the gene-editing machinery (Feng et al., 2014). Additionally, it is possible to use paired Cas9 nickases to effect more targeted single-strand breaks with subsequent homology-directed repair vs. Cas9 nuclease-induced DSBs repaired by nonhomologous end joining (Cho et al., 2014). The DNA methylation of either a 20-bp recognition sequence or the PAM, however, does not appear to affect targeting ability (Hsu et al., 2013).

Optimization of gRNA length to retain high target-site editing efficiency while minimizing off-site mutation is a promising approach for CRISPR design that lessens downstream effects (Hsu et al., 2013; Pattanayak et al., 2013). It is therefore a more preferable approach than using paired nickases because it avoids potential chromosomal rearrangements, especially when performing multiplex editing (Fu et al., 2014). Truncated guide RNAs containing 17 to 19 nt showed targeting activities equal or superior to full-length 20-nt RNA while minimizing off-target mutations (Fu et al., 2014). Further shortening of gRNA to 15 nt, however, resulted in a loss of activity. From a bioinformatics perspective, this result is somewhat surprising because a longer DNA sequence should reduce the probability of finding an identical off-target mismatch; but decreased binding energy of the truncated gRNA to DNA at mismatch sites may account for this effect (Fu et al., 2014).

Recent studies have demonstrated improvement of genome editing by using chemically modified gRNAs and engineered variants of Cas9. Stabilization of gRNAs through nucleoside phosphoramidite modification of the three terminal nucleotides at both 3′ and 5′ ends improved editing efficiency at target sites with varied effects at off-target sites as shown through deep sequencing (Hendel et al., 2015). These results suggest the opportunity to increase target site editing efficiency and decrease off-target effects through chemical modification perhaps in combination with gRNA sequence modification. Rational design of gRNA through chemical modification with *O*-Me or fluoro substitution at the sugar 2′ position, or sulfur substitution into the phosphate backbone of the nucleotide bridge, increased stability and binding affinity to target sites (Rahdar et al., 2015). Interestingly, gRNA modifications that increase target-site efficiency often show a several-fold decrease in mutation at off-target sequences relative to the target site.

In addition to modification of the gRNA, the amino acid composition of Cas9 itself can be varied to alter both PAM and target specificity (Kleinstiver et al., 2015, 2016; Slaymaker et al., 2016). Introduction of several mutations within a positively charged groove that interacts with the nontarget strand following DNA binding provides at least a 10-fold reduction in off-target activity while maintaining on-target activity for most targets (Kleinstiver et al., 2015, 2016; Slaymaker et al., 2016). Although these higher-fidelity nucleases have been demonstrated to reduce off-target activity substantially, they have yet to be validated in plants.

A variety of strategies for Cas9 codon optimization and promotor sequences have been used in studies that have analyzed for off-target mutations in plants (Table 1). Based on these limited data, there is no clear association of off-target mutations with tissue-specific Cas9 codon optimization. Off-target mutations may be somewhat more frequently observed when two tandem CaMV35S promotors are used to promote Cas9 expression, perhaps

because of a dose-dependent effect (higher RNP concentration leading to greater frequency of mismatch edits), but the trend is not clear. In all instances where a U3 sequence was used to promote the gRNA, off-target mutations were observed, but again, data are too limited at this point to clearly discern if the level of gRNA expression is a determinate for off-target effects. It may be that the gRNA expression levels will only be relevant if Cas9 expression levels are too high. High gRNA expression levels could be beneficial to ensure that all Cas9 that is expressed is properly loaded (i.e., [gRNA] > [Cas9] is preferred). The effect of codon optimization is difficult to tease out of these limited data, since differences in the reagent dose (the concentration-time effect) will confound the outcome (Feng et al., 2014).

Impact of In Vitro Cell Culture on the Genome Editing Process

One of the key steps involved in plant genome editing involves the in vitro cell and tissue-based systems required for introducing GEEN reagents (DNA-encoding Cas9 and gRNAs) into plant cells and subsequently regenerating whole plants in the genetic transformation process. Almost all plant species, except for the model plant *A. thaliana*, depend on in vitro cell and tissue culture for genetic transformation. Unlike animal cells, many types of plant cells retain totipotency and developmental plasticity in the differentiated state. These abilities allow plant cells and tissues to dedifferentiate, proliferate, and regenerate into mature plants when cultured in a phytohormone-supplemented in vitro culture environment (Skoog and Miller, 1957; Steward et al., 1964). It has been extensively documented that the in vitro artificial hormonal environment causes genetic and epigenetic changes in plant cells (Neelakandan and Wang, 2012). Genetic changes include transposable elements activation, chromosome breakage and rearrangement, or base substitution in the DNA sequence. Epigenetic changes include alteration in DNA sequence methylation levels, chromatin modification, or small RNA-mediated regulation. Most of the time, in vitro culture-induced changes do not affect phenotypes. Some off-types or variants identified in the clonally propagated populations during the process are either discarded or further advanced for breeding purposes.

Because of these in vitro culture-induced nucleotide changes in DNA sequences, it is conceivable that some PAM sites can be altered during the transformation process. Unpredicted PAM site alteration can lead to reduced efficiency at target sequences or increased activities at off-targets. For example, an originally non-PAM sequence could be mutated during the in vitro culture process and become a PAM site. Additionally, these methods may be a source of mutation that may be incorrectly ascribed to off-target genome-editing effects. Therefore, future bioinformatics design tools need to take the in vitro culture-induced mutation into consideration along with the rate of spontaneous mutation that may be occurring.

Regulatory Implications of Off-Target Effects

The regulatory climate surrounding commercialization of plants created using GEEN technologies is currently unsettled. Regulatory opinions vary among authorities worldwide (Pauwels et al., 2014; Whelan and Lema, 2015; Wolt et al., 2016) and are in a state of flux (Kershen, 2015). The suggestion (Ishii and Araki, 2016) that there is an a priori need to fully assess environmental risk of genome-edited plants in a manner similar to that currently performed for transgenic genetically modified plants is neither practicable (Jones, 2015) nor scientifically defensible (Huang et al., 2016). However, greater assurances as to the prevalence and impact of off-target genome edits will be useful for understanding the potential for unintended phenotypic effects and will increase scientific, regulatory, and public acceptance of the technology.

As regulators consider the appropriate future path for this technology, a greater understanding of unintended effects will be needed. The occurrence of off-target edits within the genome resulting from mismatches of the CRISPR does not in and of itself constitute a safety concern. However, a CRISPR mismatch resulting in editing at other than the target position is a potential precursor to an undesired phenotypic effect in a genome-edited plant. In many instances, an off-target edit will be eliminated as an undesired phenotype as the genome-edited plant is characterized in subsequent plant breeding for stability and performance over generations and environments.

Conclusion

Compared with current chemical and radiation mediated mutagenesis methods commonly used in plant breeding programs, GEEN technology offers a more precise and effective alternative for generating novel plant traits. Regulators will have increased certainty as to the degree of concern for unanticipated downstream effects originating from off-target edits if there is assurance that CRISPRs can be designed and targeted with a high degree of specificity. Improved design tools that better discriminate target and potential off-target positions, that incorporate consideration of the designed nuclease fidelity and selectivity, and that are validated will help to increase confidence for risk-based decision making (Corrigan-Curay et al., 2015).

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