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A technological and regulatory outlook on CRISPR crop editing

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Abstract

Generating plants with increased yields while maintaining low production and maintenance costs is highly important since plants are the major food source for humans and animals, as well as important producers of chemicals, pharmaceuticals, and fuels. Gene editing approaches, particularly the CRISPR-Cas system, are the preferred methods for improving crops, enabling quick, robust, and accurate gene manipulation. Nevertheless, new breeds of genetically modified crops have initiated substantial debates concerning their biosafety, commercial use, and regulation. Here, we discuss the challenges facing genetic engineering of crops by CRISPR-cas, and highlight the pros and cons of using this tool.

Keywords

CRISPR specificity; genetically modified organisms; regulatory agencies

1 Introduction

“An army marches on its stomach” Napoleon Bonaparte purportedly said over the Battle of Waterloo, realizing that many wars are settled in favor of those with available food supplies. Historical events such as the “Great Famine” in Ireland, spanning from the mid-1840s to mid-1850s, led to the death of approximately one million citizens as well as to the emigration of almost two million people.¹ This is a stark example of how an entire country's demographics can be destabilized due to crop unavailability.

Predictably, farmers, governments, and scientists are constantly striving to improve crop yields. Traditionally, breeding techniques were successfully used to improve the quality and quantity of crops. The use of pesticides and fertilizers in the past decades dramatically improved crop yields. However, these chemicals also pollute the environment and can negatively affect the quality of consumed food. Alternative, more environmentally friendly solutions, such as genetic engineering (GE) of crops, offer a facile, non-toxic, and efficient approach for improving plant yields.

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Coupling sequence specificity with the ability to induce breaks in the DNA is the hallmark of modern genetic engineering. Modern approaches utilize guided nucleases, that can introduce breaks in the DNA and, when coupled with a desired DNA binding domain (DBD), they can be directed to a specific target area. Subsequently, stimulating the host's DNA repair mechanisms; leading to mutation, and potentially complete loss of function of a certain gene of interest. Hitherto, four types of engineered nucleases have been developed and used for genome engineering: meganucleases, Zinc finger nucleases (ZFNs), transcription activator-like effector nucleases (TALENs), and CRISPR-Cas9 and orthologs. All methods have been applied for genome manipulations, however, the first three methods all have closed architecture that is difficult to manipulate, rendering them laborious and expensive to manipulate and “customize.” Moreover, CRISPR systems rely on guide-RNA (gRNA) and ribonucleotide-DNA complexes for target recognition and subsequent cleavage rather than protein-DNA complexes. This feature also renders CRISPR more versatile, easy to produce, and less costly.²

Genome alteration may occur naturally, by mating for example, or by genetic engineering using gene-editing tools (eg, CRISPR-Cas9). An organism is defined as a genetically modified organism (GMO) when the alteration to the DNA occurs via GE rather than in a natural way. The CRISPR-Cas system is the most effective tool currently available for genetic engineering. This system originated in prokaryotes as a defense system against viruses and other parasitic elements. CRISPR-Cas systems are found in the majority of archaea and in many bacteria.^{3,4} The systems are diverse and the most recent classification posits that there are two classes that are further divided into six types.^{5,6}

Currently, the Type II CRISPR-Cas9 is mainly used for genetic engineering. This system was shown to function as an RNA-guided enzyme that cleaves DNA and generates double-strand breaks (DSBs).^{7–10} The system is comprised of Cas9, single guide RNA (sgRNA), and trans-activating CRISPR RNA (tracrRNA) which are RNA structures essential for the assembly of the effector complex and for DNA recognition.¹⁰

Genome editing requires generating a precise break in the target DNA. CRISPR-Cas9 induces DSBs following the recognition and binding of a specific target. The breaks are induced by Cas9's two catalytic domains named RuvC and HNH, which cleave the DNA at a specific position determined by the guide RNA, leaving a blunt end.¹⁰ DSBs are commonly repaired by homology directed repair (HDR), or by non-homologous end-joining (NHEJ).¹¹ HDR relies on sequence homology to repair DNA breaks. Templates with homologies surrounding the cleaved region can be introduced to the cell, as either dsDNA or ssDNA, resulting in precise alterations of the loci. Alternatively, the NHEJ pathway operates in a template-independent manner, usually leading to insertion or deletion mutations (indels) that disrupt the gene's reading frame or disturb various essential sequences such as those of promoters and other regulatory sequences.¹² Numerous studies have used this tool to edit various organisms. For thorough recent reviews, the reader is referred to the following articles.^{13–15}

Biotechnological innovations, such as harnessing CRISPR-Cas for genetic engineering, have been readily adopted by many academic and industrial research groups. Indeed, the

agriculture and food industries have quickly implemented the CRISPR-Cas editing process.^{16,17} Specifically, CRISPR is used to produce genetically modified plants, which will hopefully be used to greatly improve crop yields and other traits that are found in commercially used GM crops. Such as those engineered to withstand drought stress,¹⁸ and viral attack.^{19,20} However, there are still many obstacles in producing GE plants, particularly in obtaining regulator, and public opinion approvals of such products and determining whether they are GMOs or not. We will focus on utilizing CRISPR editing for producing plant GMOs and the regulatory framework of GMO crops and its derived food.

1.1 CRISPR-Cas for generating plant GMOs

Shortly after deciphering the major molecular mechanisms governing CRISPR-Cas, and repurposing it as a molecular tool,¹⁰ two groups demonstrated that Cas9 can be used for editing eukaryotic human cells.^{21,22} Subsequently, CRISPR-Cas was used to edit various organisms or cells from organisms such as rodents,^{23,24} fish,²⁵ flies,²⁶ worms,^{27,28} yeast,²⁹ bacteria,³⁰ bacteriophages,^{31,32} and plants.^{33,34} Additional research groups independently showed that CRISPR-Cas9 technology can efficiently edit genomes of various crop species, such as tomatoes, rice, and wheat.^{35–37} However, editing efficiencies were relatively low. Nevertheless, this demonstrated the versatility and potential of gene editing in plants using CRISPR-Cas technology and it was further developed and improved. For example, various vector systems were constructed for improved expression of the CRISPR-Cas components and higher editing efficiencies. Many Cas9 genes have been codon optimized; a strategy that, in most cases, resulted in improved efficiency.^{37–40} Moreover, promoters driving the expression of vectors encoding the CRISPR system have also been altered. Promoters such as Cauliflower Mosaic Virus (CaMV) 35S, and those of ubiquitin gene derived from various species were shown to be sufficient for Cas9 expression. Vectors for sgRNA expression were also improved and expression is commonly driven by the U3 or U6 snRNA promoters.^{35,36,41} Other approaches exist as well,⁴² although sgRNA U3/6 driven expression coding sequences are relatively small, hence are easy to produce and express in the target cell.

To facilitate genomic changes, the constructs encoding the CRISPR-Cas9 components must first be delivered into the target plant cell. The *Agrobacterium*-mediated transformation method is efficient for many plants. Thus, many applications use this method for delivery.^{36,39,40,43} However, inserting foreign DNA into plant cells poses regulatory challenges in the context of producing GMOs for food production. Furthermore, *Agrobacterium*-mediated transformation is not the most efficient method for all plant types, therefore, different groups demonstrated additional delivery methods (eg,^{44,45}).

As mentioned, CRISPR-Cas technology has been utilized in plants for several years and it is continuously employed in new species and targets. For example, powdery mildew locus O (*MLO*) genes were targeted in tomatoes using CRISPR-Cas9.⁴⁶ *MLO* genes encode proteins conferring susceptibility for *Oidium neolycopersici*, a common fungal pathogen, which is the cause for powdery mildew disease in tomatoes.⁴⁷ Out of 16 *MLO* genes, *SIMlo1* was chosen as a knockout target because this gene is the major contributor to the pathogen's vulnerability. The engineering procedure used a previously described method, where two sgRNAs guided a Cas9 complex to target different regions of the same gene.^{35,48,49} This

strategy generated a deletion of the area between the two targets. After confirming by PCR that the deletion occurred, the engineered plant was self-fertilized, generating progeny that do not carry the DNA encoding the CRISPR-Cas cassette. As a result, the transgene-free mutant plant with a deletion of one susceptibility gene, demonstrated resistance to the *Oidium neolycopersici* pathogen.

Self-fertilization is not possible in plants that reproduce asexually, and therefore, for these types of plants, approaches for generating transgene-free plants should not rely on DNA-based vectors to deliver the CRISPR components. Kim et al indeed refrained from delivering a DNA cassette, and instead delivered pre-assembled CRISPR-Cas9 ribonucleoproteins (RNPs) into various plant species.⁴⁵ Similar to the work executed in human cells and in *Caenorhabditis elegans*,^{50,51} they purified Cas9 proteins and mixed them with sgRNA, targeting several genes of arabidopsis, tobacco, and rice. This in vitro assembled complex was incubated with protoplasts derived from these plants and the target locations were subsequently tested for mutations. Success rates of this engineering procedure varied from 8.4% to 44% in the tested plant species. Since no DNA is used to deliver the construct, this strategy may help relieve regulatory concerns of plant editing.

CRISPR-Cas based technologies has been extended beyond gene editing, and methods that do not alter the sequence of the DNA have been developed. By inducing mutation in the two nuclease domains of Cas9, it is possible to abolish the cleavage activity while retaining the ability to bind a specific target. The resulting deactivated Cas9 (dCas9) can be further engineered for repression or activation of transcription (termed CRISPRi or CRISPRa for interference or activation, respectively),^{52–55} epigenome editing,^{56,57} and it has also been coupled to various inductions such as by light⁵⁸ or riboswitch.⁵⁹

1.2 Regulatory approvals of CRISPR-engineered GMOs

Transgene-free procedures for generating GMOs are desirable because they can circumvent regulatory obstacles. Indeed, in 2016, the United States Department of Agriculture (USDA) decided that genetically modified mushrooms that used CRISPR-Cas9 technology are exempt from the USDA's GMO regulations since these mushrooms do not fall under the requirements for these regulation.⁶⁰ The white button mushrooms, *Agaricus bisporus*, were engineered with small deletions in genes encoding polyphenol oxidase, an enzyme involved in the browning process of the mushroom. The mutations reduced the enzyme's activity, resulting in a more appealing exterior and prolonged shelf life. No antibiotic markers were used for selection during the mushroom's production, and no traces of the CRISPR-Cas9 system employed in the process were left, as confirmed by PCR and Western blot analysis. Thus, these mushrooms “escaped” USDA regulations, paving the way for other GMOs to follow.⁶¹

A letter along the same lines was issued in response to an inquiry made by the American company, DuPont Pioneer.^{62,63} DuPont utilized CRISPR-Cas9 to knock out the Wx1 gene in corn, which encodes a synthase producing the polysaccharide amylose. This modification resulted in a CRISPR-edited corn containing starch made of a different polysaccharide, amylopectin, a commodity used in several industries. The letter posted by the USDA concluded that this GMO also escapes the agency's regulations.

Overall, these examples indicate that the regulatory bodies do approve different GMO products that were engineered using the CRISPR-Cas system, and are more inclined to do so as long as the system is removed from the engineered organism. This approach is rational, since the endpoint product is identical to a product made in the traditional way, which would have taken significantly more resources, labor, and time.

1.3 Regulatory agencies and policies on GMOs

In the United States, the USDA is not the only agency that regulates GMOs. The US regulatory framework also includes the Food and Drug Administration (FDA) and the Environmental Protection Agency (EPA). Depending on the GMO in question, approval of one to three agencies may be required. For example, the EPA oversees the safety of pesticides and therefore, GMOs that are engineered to overcome certain pesticides are regulated by the EPA. Products that are intended for human consumption are likely to be regulated by both the USDA and the FDA.⁶⁴ Insertion of a gene encoding properties that support drought resistance is not under the EPA's jurisdiction but might be under the jurisdiction of the FDA and the USDA. US regulations and policies are generally technical and are based on the engineered trait and the intended use of the product.⁶⁵ Primarily, safety and efficacy are considered, while other implications, such as moral, cultural, and socioeconomic issues have little to no impact. Moreover, the FDA concluded that no substantial differences exist between genetically engineered or traditionally bred crops. Therefore, it is not within the agency's power to mandate any additional labeling.⁶⁶

The European Union (EU) relies on the European Food Safety Authority (EFSA) to scientifically review the safety and environmental impact of GMOs. In general, scientific agencies of the different member states cooperate with the EFSA to provide a scientific opinion. Final decisions are made by the European Commission. Strong public opposition exists in some member states, making the approval of GM crops difficult. Thus, even a crop approved by the European Commission is not likely to be cultivated in all of the member states. In the EU, GM foods must be properly labeled by law.

The different regulations, as well as cultural perceptions, governing different countries, reflect the diversity regarding GMO approvals. There are different procedures for the regulation and oversight of GM crops and food produced from these crops. The different approaches by governments represent different responses to the public opinion and the scientific community. The policies also reflect diverse cultures, environmental conditions, and often also political pressure and interests of various groups such as farmers, agriculture companies, and environmental activists or agencies.

Adding to the complexity of regulation policies is the emergence of technologies that enable gene manipulation without changing the sequence of the DNA, and GE plants that lack transgenes. In theory, it should be easier to overcome regulatory hurdles using CRISPRi for example, rather than conventional CRISPR-Cas9 to knockout a gene. Technically, plants made this way should not be regarded as GMOs because no alteration to the genome occurred. However, the drawback of such methods, in this context, is that for the most part they require the presence and constant expression of the CRISPR components. So, practically, it is likely that they will be considered GMOs due to the process, and especially

due to the fact that they contain exogenous DNA. An exception to this might be epigenome techniques that, in theory, could be expressed transiently, then selected against in progeny while maintaining the change conferred. To our knowledge, there is no precedence for this. Overall, these methods still require further development in plants.

The question whether plants made this way are GMOs varies depending if we examine the process or end-product.⁶⁷ suggest that regulation should move from process-based to product-based. Meaning, instead of assessing the process, which is usually a long expensive process, risks should be assessed based on the end product, after surveying off-target mutations. Moreover, opinions published by the USDA state that some mutations made by ZFNs, meganucleases, TALENs, and CRISPR that induce the NHEJ pathway are beyond its regulatory scope.⁶⁸ Taken together, this suggests that CRISPR technology can be used to make plants that are not regarded as GMOs.

1.4 Widespread GMO utilization

Despite difficulties in approval procedures, as well as in public opinion acceptance, the commercial use of GM crops in the US started over two decades ago and rapidly became a common practice. For example, in the year 2000, 54% of the cultivated areas in the US dedicated to soybeans were of GM soybeans. This number steadily increased and in 2007 it reached over 90%. GM corn and cotton have shown similar trends.⁶⁹

Most commonly, GM crops are herbicide-tolerant (HT), insect-tolerant (Bt), or both. HT crops are engineered to withstand potent herbicides that would otherwise kill both the crops and weeds. These plants are most commonly engineered to resist the application of glyphosate, a potent herbicide that acts against a wide range of weeds. Bt crops commonly contain a gene from the bacterium *Bacillus thuringiensis*, encoding a toxin that perforates the gut of several insects, slowing their growth, and eventually leading to their death.⁷⁰ Planting of Bt crops allow less insecticide use and improve yields.

The aforementioned traits are the most common in commercial use, yet other traits exist, or being developed, and are aimed toward wide-spread commercial use. These mainly include withstanding fungi Antony Ceasar and Ignacimuthu, 2012 and viral infection. In addition to traits involved in increasing crop yields, GE is also employed in crops for other purposes. For example, producing increased amounts of nutritional elements such as vitamins, proteins or pharmaceutical products,⁷¹ changing the color of commercially sold flowers,⁶⁵ pollination control systems, and traits aimed at improving the quality of the product in terms of appeal and longer shelf-life. Overall, GMOs are widespread, and the increasing trend of their use will undoubtedly continue.

1.5 Hurdles and solutions in producing CRISPR-based GMOs

1.5.1 CRISPR-Cas specificity—Specificity is an integral part of efficient genome editing, particularly when used as a tool for GMO production, which merit strict regulation. Unfortunately, CRISPR-Cas9 tolerates a certain degree of mismatches between the sgRNA and the target,^{23,72} leading to off-target editing. Some collateral effects of CRISPR-Cas use can also be attributed to the delivery vectors. It was hypothesized that such vectors, used to

deliver Cas9 components, can integrate into the cell's genome via recombination or can be inadvertently used by the cells as a template for DNA break repair.⁵¹ While most of the data comes from human cell applications, research in plant cells also suggest off-target mutations in some cases.⁷³

To alleviate this problem, several strategies have been developed such as using pre-assembled ribonucleoproteins (Cas9 + sgRNA) instead of extracellular DNA. This reduces CRISPR-Cas activity in the cell as its components are not constantly produced, and also alleviates the collateral effects attributed to the delivery vectors. Another method used is executing whole genome sequencing, Digenome-seq, or other highly sensitive methods for the exclusion of GMOs with off-target mutations.^{74,75} These sequencing methods validate that only the desired changes have been introduced into the GMO. A third method used is choosing highly specific target sequences to reduce off-targets. This is achieved by using simple and accessible tools such as BLAST or specific databases.⁷⁶ Last, to drastically reduce the off-target activity, we can use higher fidelity Cas9 or other Cas9 orthologues with higher fidelity, or shorten the gRNA.^{77,78,79,80,81}

1.5.2 Public unacceptance of GMOs—Poor public endorsement of GMOs is a major problem facing GMO utilization and development. A negative attitude toward GMOs is usually associated with lack of sufficient knowledge, and lack of trust in developers and regulators.^{82,83} It is therefore essential to raise public awareness by educating the public regarding the different breeding techniques and technologies employed for generating GMOs. Such transparency enables consumers to make informed decisions based on their rational judgment. Transparency will also promote science and increase the public's support. This in turn would lead to increased product demand, and subsequently, encourage scientists and biotechnology companies to invest in research and development. Though cultural and socioeconomic factors have a place in the discussion, understanding the technology, and the science behind it, is the foundation for rational decision-making.

In this context, we should mention key findings of a report from the National Academies of Sciences, Engineering, and Medicine examining the negative and positive effects of GM crops.⁶⁵ The report found that there is almost no evidence to connect GM crops to negative environmental effects. Conversely, some benefits were found, such as reduced exposure to pesticides. Regarding humans, there was insufficient evidence to indicate any risks. Animal studies showed no health differences when eating GM or non-GM foods. The committee however noted, both with regards to the environment and human health, that some of the tools available for risk assessment are limited and that long-term effects are difficult to measure and interpret.

1.5.3 Ethical issues—Despite the relative simplicity and accessibility of the CRISPR-Cas system for GM, generating GMOs is still expensive. Producing, testing, and bringing to market new crops is something that usually requires substantial amounts of capital investment and the backing of large companies or institutions. Consequently, society must take into account that GMO crops may harm small scale farmers because they cannot compete with the more expensive patented strains that have higher yields.

Developing countries, experiencing lack of crops or lack of certain essential nutrients in the crops grown, may benefit from increased yields, crop resistance to drought and disease, and plants engineered to produce more vitamins etc. They could also benefit from crops with reduced fungal toxins contamination, a prevalent issue in developing countries.⁸⁴

Agriculture is instrumental in developing countries, yet commercially, the focus is largely on Western countries. Because the main discussions on GMO development takes place primarily in the US and the EU, the tremendous benefits that developing countries can derive from GMOs are occasionally not taken into account. This issue should also be considered when making decisions regarding GMOs.

The issues communicated here are not all specific to CRISPR and most of them precede the technology. Specifically, ethical issues, public unacceptance, how to regulate GMOs, and even what is a GMO, are all issues that existed since GE plants emerged. A CRISPR-specific issue is intellectual property (IP). Although some advancements have been made in court, it is yet to be determined who owns the patent for CRISPR-Cas9 genome editing in eukaryotic cells (For a better understanding of the IP dispute the reader is referred to.⁸⁵). IP disputes are a concern since they have an integral role in the development and implementation of CRISPR technology. However, this has driven the discovery of Cas9 orthologs, and exploring the utilization of different, non-Cas9, systems for gene editing. CRISPR technology entered an existing dialog and affected it because of its versatility, which sometimes challenges conventional definitions and can circumvent regulation, and because it advanced scientific understanding and capabilities regarding mutagenesis, making it more precise, quick, and accessible.

2 Summary and Prospects

Over the past few years, CRISPR emerged as an instrumental gene-editing tool due to its robustness, efficiency, and wide range of applications. CRISPR technology is paving the way for more precise cellular investigations by making it easier to change single genes or even single nucleotides, rapidly, and accurately. CRISPR can be used to easily silence or activate genes of interest, thus helping in elucidating their roles in specific pathways⁵⁵ or to sensitize bacteria to antibiotics,⁸⁶ demonstrating the versatility of CRISPR. With respect to plants, CRISPR-based technology is gradually avoiding using transgene DNA. It also achieves precise and specific validated modifications of the target gene without collateral effects, thus easing regulatory concerns.

The accessibility of CRISPR technology facilitates an increasing amount of research. This can be seen by the profusion of data and tools that have emerged despite that fact that CRISPR surfaced only several years ago. It is easy to envisage that many more advancements and novel applications will be developed in the near future. Ethical issues should be discussed, and the public should be exposed to the technology as much as possible, as we believe that regulatory decisions should be based on transparent scientific facts.

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