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Applications of Crispr/Cas System in Plants

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ABSTRACT

Every year, the world's population grows, and to sustain this enormous population, food consumption must likewise massively double. However, because of unpredictable environmental fluctuations, plants are unable to produce the necessary amount of food, and several variables have negatively impacted their growth. Abiotic stresses like drought and salinity stress have already caused too much destruction in crop yield and are expected to decrease the yield in coming years. Also, biotic factors cause a severe threat to plant production. Conventional strategies are not much effective in controlling these disasters in plants. Biotechnological innovations have much potential to modify the tolerance mechanism in plants or engineer another process to make tolerant varieties. CRISPR/Cas technology has emerged as a promising single or multiple-site-directed mutagenesis tool. The CRISPR Cas technique alters the key genes that code for the enzymes that support metabolic pathways. The end goal is to raise fruit quality. It has advantages compared to conventional random mutagenesis methods due to its high efficacy, precision, and low risks of off-target activities. In the present study, CRISPR/Cas technology potential has been described as modifying plant genomes for high yields under various biotic/abiotic stresses.

Key words: Abiotic Stresses, Crispr/Cas9 System, Food Security

INTRODUCTION

There is a vast increase in the world population, and this doubling is expected to be more notable in the year 2050 when it is estimated that the world population will be nine billion. It needs time to have a sustainable and efficient agriculture system to meet the hunger demands of such an increasing population. Many challenges, like biotic and abiotic stresses, exist to have a sustainable agriculture system (Bray, 2000; Raza et al., 2019).

Genetic engineering of different plants and the interpretation of resulting phenotypes is a vital strategy to identify the function of specific genes. Various genome engineering tools have been identified to target the genome of different organisms with great precision and accuracy (F. Zhang et al., 2011). Genetic engineering is a technique to insert foreign gene sequences into a plant's genome at a specific location to develop transgenic (Haroon et al., 2022).

Now, it becomes possible for researchers to understand the pathological processes of various diseases and their causes behind. They have controlled various diseases using different genome engineering tools (Colwell, Norse, Pimentel, Sharples, & Simberloff, 1985).

However, the main focus of the researchers of this scientific era is to bring site-directed mutations in the genome of living systems more precisely and efficiently. RNAi, Tilling, and Reverse Genetics are different homology-directed techniques and have been used for many years. However, there are certain drawbacks to using these techniques, such as random integration or off-target effects, and after some time, the expression of transgene recovered. Recently some new approaches such as ZFNs, TALENs, and CRISPR/Cas have been developed for precise and targeted genome engineering for crop improvements (Reis, Hornblower, Robb, & Tzertzinis, 2014).

CRISPR-Cas9 is an adaptive immune system in archaea and bacteria against invading plasmids or viral DNA sequences (Jinek et al., 2013). The bacterial CRISPR-Cas9 system comprises CRISPR loci, two non-coding RNAs, and Cas9 endonuclease.

The critical functional component of this system is the Cas9 endonuclease.

Foreign DNAs are integrated into CRISPR loci during spacer acquisition, followed by the transcription and processing of the loci into mature CRISPR RNAs (crRNAs).

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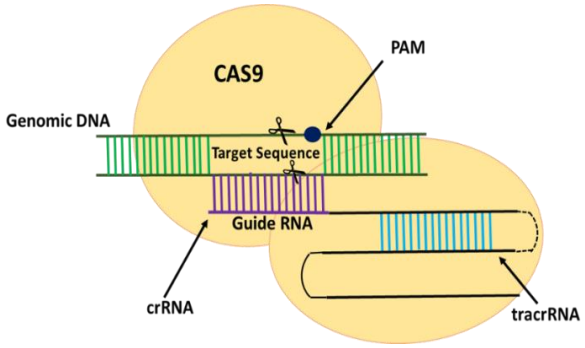

Fig 1: Main components of CRISPR-Cas9 System.

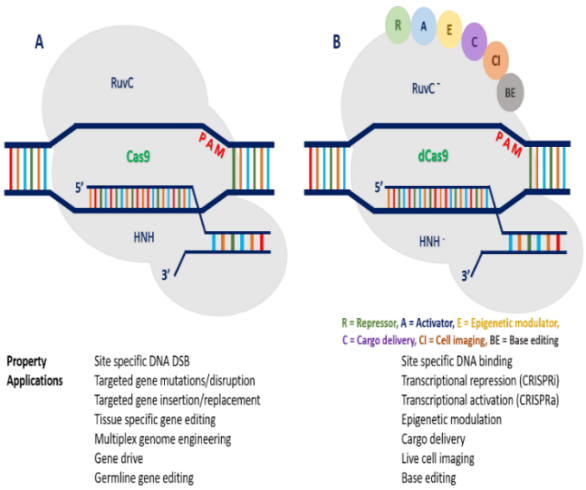

Fig 2: Assembly of CRISPR-Cas9 complex.
(A) Critical features of Cas9-gRNA complex.
(B) Key features of catalytically dead Cas9-gRNA complex.

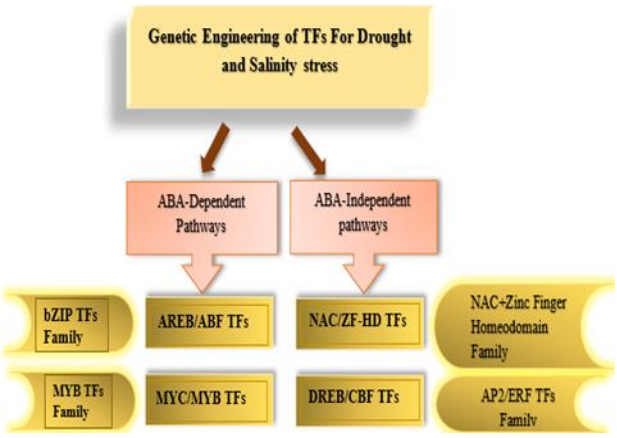

Fig 3: Genetic engineering of Transcription factors (TFs) for drought and salinity stress.

These mature crRNAs then guide the Cas9 endonuclease protein to scan and degrade complementary DNA of invading plasmids or viruses. Therefore, the CRISPR-Cas9 system provides the host with acquired and heritable resistance (Marraffini & Sontheimer, 2008).

Molecular biologists have rapidly developed the CRISPR-Cas9 system into a powerful genome editing tool in applied plant biotechnology. Due to the cheap and quick method of inducing site-specific genetic modification has rapidly become a method of choice (Doudna & Charpentier, 2014; Shen *et al*., 2017).

In plants, it has been widely adopted for breeding desirable traits. Multiple gRNAs for several target genes can also be assembled to simultaneously edit multiple loci (Gaj, Gersbach, & Barbas III, 2013)(Wang *et al*., 2018).

a. Resilience to abiotic stresses:

Many challenges, like biotic and abiotic stresses, exist to have a sustainable agriculture system (Bray, 2000; Raza et al., 2019). Abiotic stresses are the harmful effects of non-living and environmental factors on living things like plants (Acquaah, 2009).

These are drought, salinity, frost, high temperature, and other harsh environmental stresses (Zafar et al.,2022a; Zafar et al., 2020a). Among these, salinity and drought stresses are interconnected as they both affect the plant in a somehow similar fashion. (Saxena, Kumar, & Tomar, 2019). According to FAO, 800 million hectares of land are distressed due to salinity worldwide, and a considerable part of the land is susceptible to salinity (Hasegawa, Bressan, Zhu, & Bohnert, 2000) (Burke, Brown, & Christidis, 2006). It is predicted that 1-3% of the land today and 30% in 2090 will face longer drought stress(Burke et al., 2006).

To have a complete check and balance on Na+ and Cl accumulation, scientists have isolated certain Na^+/H^+ ion antiporters as they play an essential role in maintaining ion homeostasis at cellular levels in plants.(Serrano & Rodriguez-Navarro, 2001) Na^+/H^+ antiporters mobilize the flux of Na^+/H^+ across the membrane and exchange the Na^+ and H^+ ions (Horie et al., 2005; H. Shi, Quintero, Pardo, & Zhu, 2002). SOS1 gene catalyzes the redistribution of Na^+ between root and shoot. SOS2 and SOS3 kinases present in Arabidopsis catalyze the activity of SOS1 ion exchangers (Qiu, Guo, Dietrich, Schumaker, & Zhu, 2002; Quintero, Ohta, Shi, Zhu, & Pardo, 2002). Overexpression of SOS1 and AtNHX1gene in Arabidopsis resulted in increased tolerance to salinity and decreased accumulation of Na^+ ions in the leaves (Apse, Aharon, Snedden, & Blumwald, 1999).

Transcription factors (TF) in plant-resistant mechanism

Transcription factors play an essential role in activating stress-related genes to cope with adverse environmental conditions(Fowler & Thomashow, 2002; H. Wang, Wang, Shao, & Tang, 2016) (Umezawa, Fujita, Fujita, Yamaguchi-Shinozaki, & Shinozaki, 2006).

It is estimated that 5.9% genome of Arabidopsis thaliana codes for 1,500 TFs (Udvardi et al., 2007; Umezawa et al., 2006). The transcriptomic data of Arabidopsis thaliana shows that various pathways respond either in an ABA-dependent or ABA-independent manner to abiotic stresses(Lata, Yadav, & Prasad, 2011).

By using CRISPR-Cas9 gene editing in indica rice cv.MTU1010, a mutant allele of a gene that shows drought and salt tolerance produced. Targeted regions are two gRNA of the DST gene that contribute to the protein-protein association. After successfully deleting 366 bp between gRNA, create mutant alleles, and choose the mutant homozygous allele. This mutant allele is named DSTΔ184-305 due to the deletion of amino acid residues. This mutant allele introduces in the indica rice through CRISPR Cas 9 method. Due to this mutation, leaves produce that are wide and contain less stomatal density.

Table1: Examples of successfully developed Abiotic Stress Tolerant Crop using CRISPR-Cas technology

Target sequence	Type of target sequence	Type of edit	Species	Results	References
Oryza sativa bilateral blade senescence 1 (BBS1)	Gene	Deletion	Rice	The absence of a targeted gene enables the plant to be sensitive to drought stress	(M. Zhang et al., 2018)
4(OsPT4)	Gene	Deletion	Rice	Decrease the up-take activity of arsenic from rice	(Y. Ye et al., 2017)
SIMAPK3	Gene	Deletion	Tomato	Plants susceptible to drought stress	(L. Wang et al., 2017)
ARGOS8	Promotor	Promotor replace	Maize	Increase expression of the gene	(J. Shi et al., 2017)
Oryza sativa 9-cisepoxycarotenoid dioxygenase	Gene	Mutation	Rice	Susceptible to drought and salinity	(Huang et al., 2018)

So ultimately, leaf water retention increases during dehydration stress. Thus, the DST mutant alleles produced throughout this study would help in indica rice cultivars to improve drought and salt tolerance and grain yield (Kumar et al., 2020). It is proved that CRISPR dCas9HAT is a powerful biotechnological tool through positive control of AREB1 to boost drought stress tolerance (Paixão et al., 2019).

Both for its tremendous nutritional and commercial values, tomato is a prevalent crop and is sometimes used to research gene function (Li et al., 2019).

With the help of CRISPR-Cas 9 mediated genome editing technology, an S gene known as S1AGAMOUS-LIKE 6 (SIAGL6) in tomato targets improves fruit setting in heat stress conditions (Klap et al., 2017).

OsAnn3 gene from rice plant knockout with CRISPR-Cas9 technique and mutant lines developed. It demonstrates the role of the annexin gene in cold stress tolerance in plants (Shen et al., 2017). T1 mutant lines produce that contain cold tolerance phenotype from T0 biallelic mutants. Results explained that relative electrical conductivity increased and the survival duration of T1 lines decreased compared to wild-type plants. In the end, results demonstrate that OsAnn3 is involved in the cold tolerance of plants (Shen et al., 2017).

b. Resilience to Biotic stresses

Insects are a severe threat to agriculture sustainability (Zafar et al., 2020b; Zafar et al., 2022b). More than 10,000 species of insects are threatening different field crops (Dhaliwal, Jindal, & Dhawan, 2010). Various novel technologies have emerged to genetically modify crop plants to confer pest resistance to combat yield losses due to chewing insects. The delta protein from *Bacillus thuringiensis* has been exploited commercially to develop resistant plants against chewing insects (James, 2011; Wu *et al*., 2008;) As a result, the insect distribution pattern in the field has changed due to the wide adaptation of genetically modified crops and reduced utilization of insecticides (Tabashnik *et al*., 2008; Zhang *et al*., 2011).

The adverse effects of chemical pesticides can be reduced by enhancing insect resistance in crop plants. So, biotechnology-based approaches are appealing alternatives to control pest populations because it seems impossible to develop resistance against insects through conventional plant breeding approaches. The reason is the unavailability of insecticidal genes within the crossable plant species. Therefore, alternative strategies can be adopted to control infestation caused by phloem feeders. Biotechnology-based strategies used previously against aphids include incorporating insecticidal or anti-aphid genes in different plant species and silencing aphid-responsive genes through RNAi.

With the development of plant transformation techniques, various aphid-resistant genes have been introduced into the plants. Studies on the development of aphid resistance mainly focused on the characterization of genes that can confer resistance in plants. The development of Bt crops is the triumphant story of genetic engineering, but it is well known that these crops are effective only against *Lepidopterans, Dipterans*, and *Coleopterans*. The Bt toxin does not affect the insects belonging to the order *Hemiptera*, which are also very destructive pests of various crops and cause substantial yield losses by directly ingesting the phloem sap or indirectly transmitting different types of viruses. Earlier research studies have revealed that lectin genes such as *Galanthus nivalis* (GNA), *Allium cepa* (ACA), and *Pinella ternata* (PTA) exhibited anti-aphid ability not only against aphids but also other insects of order *Hemiptera*. These genes have been found to interfere with insect developmental processes resulting in reduced growth rate, larval fecundity, and the number of larvae produced per aphid is also lowered. The RNA-guided Cas9 system can help rewrite genomic information, improving crop production and increasing resistance against viral and bacterial diseases. It can be achieved by inducing DSBs concurrently at multiple sites in DNA and is very useful in undesirable knockout genes.

Multiplex genome engineering fully describes the practical application of gene knockout with this newly emerged system, which requires Cas9 endonuclease with multiple sequence-specific guide RNAs (gRNAs) (Cong and Zhang, 2014;).

The current study aimed to demonstrate the application of the CRISPR-Cas9 system to investigate its potential to develop plant resistance to Cotton leaf curl disease (CLCuD), a devastating disease of cotton in Pakistan. Both coding and the non-coding virus DNA sequences were targeted by RNA-guided Cas9 complex. Targeting of the CLCuV genome at four different sites resulted in a significant reduction of virus titer and reduced infection. Furthermore, the multiplex Cas9-gRNAs vector could simultaneously target six coding genes of CLCuV, resulting in effective cleavage of the

CLCuV genome at three different positions. A significant reduction in virus accumulation and symptom severity has been demonstrated. The study has proved that with a multiplex approach, it is possible to target multiple viruses and satellites simultaneously, a key advantage to controlling CLCuV, which is a case of mixed infections by several viruses.

Tomato is susceptible to the attack of nematodes, weeds, and fungal, viral, and bacterial pathogens. Bacteria are responsible for wilt, canker, and speck disease development in tomatoes (Poudel & Neupane, 2018). Nematodes cause tomato root-knot diseases when they cause infection (Nono-Womdim, Swai, Mrosso, Chadha, & Opena, 2002). Fungal attack results in the development of wilt, early and late blight diseases of tomatoes. All these pathogens are responsible for 77.7% of yield losses (Zalom, 2002).

In the case of fusarium wilt disease of tomato, XSP10 protein has been identified in the xylem sap of tomato (Krasikov, Dekker, Rep, & Takken, 2010). XSP10 protein resembles lipid transfer-like proteins and is found in high amounts in xylem vessels. When the *Fusarium oxysporum* attacks the xylem vessel, the amount of this protein tends to decrease, showing that the pathogen has a specific interaction with this protein (Rep *et al*., 2003). Through the mutation of XSP10, this protein will no more be able to support the infection of *Fusarium oxysporum* in tomatoes (Krasikov *et al*., 2010).

Although conventional control strategies are helpful, many limitations are associated with their use. These field efforts are costly, and fungicides affect the human body, environment, and other plant-beneficial microbes in the soil. Due to the low penetration power of fungicides, the soil-borne *Fusarium oxysporum* remains active, and the pathogen develops resistance against these chemical applicants. Along with these disadvantages, trait fixation through traditional ways requires many generations, and there are many chances of escaping that trait in the future. To control disease, alternative efforts with increased efficiency, low cost, and less harm to the environment are required.

Science and technology have always aimed to develop new machines, chemicals, and varieties to improve agriculture. In the scientific era, biotechnology provides all the tools to genetically modify the genomes of crops. Different approaches are transgenics, recombinant DNA technology, omic approaches, modern breeding techniques like reverse breeding and intragenic/Cisgenesis, QTL (Quantitative Trait Loci), NGS (Next Generation Sequencing), and genetic engineering. These techniques have significantly improved the genetic makeup in agriculture for desired characteristics.

Due to the advent of molecular biology, more recent technologies have been developed that are used for targeted editing of the genomes to get desired phenotypes precisely to control biotic and abiotic factors and control diseases. RNA interference and marker-assisted selection are efficient biotechnological tools.

Among many site-specific tools, CRISPR/Cas9 system is a promising and competent technology for achieving multipurpose tasks using plant repair mechanisms. Through genetic studies, many susceptible host factors have been identified, like the XSP-10 protein of tomato for fusarium oxysporum. The XSP-10 protein provides an interaction site to the pathogen in the xylem vessel of the tomato and supports the spread of the pathogen to the whole plant through this vessel and upsets the water and minerals distribution of the tomato. This blockage of the tomato vascular system produces yellowing and wilting symptoms. In the current study, XSP-10 was mutated through CRISPR/Cas9 technology to develop resistance against f. oxysporum in tomatoes. The sgRNA was designed using specific parameters against XSP-10 from CRISPR direct and CRISPR-P. Different plant breeding technologies have been used to develop crops to control pathogens, but these methods are not sufficient because they are laborious and time-consuming. Chemical control may harm health and cause environmental risks. RNA interference is a helpful technology for the improvement of crops. It is undoubtedly an eco-friendly technology, but it has the disadvantage of non-specificity of reagents, variability, and incomplete knockdowns. *Pseudomonas syringae* is a rod-shaped, Gram-negative bacterium with polar flagella (Krzymowska et al., 2007). This pathogen affects a wide variety of plants. To control Pseudomonas syringae, different genes have been identified. DMR6, a member of the oxygenase family, has been identified in Arabidopsis against broad-spectrum diseases. Downy mildew resistance6 gene is a Fe (II) Dependent 2-Oxoglutarate incorporates in resistance against many bacterial and oomycetes (de Toledo Thomazella, Brail, Dahlbeck, & Staskawicz, 2016). DMR6 functions in oxidoreductase activity with the incorporation or reduction of molecular oxygen. It is also involved in the flavonoid biosynthesis process. Paralogs of DMR6, DLO1, and DLO2 (DMR6-like Oxygenase), have been identified in Arabidopsis, and these paralogs show high co-regulation with *DMR6*. A low level of resistance against *H. arabidopsidis* has been shown when *dol1* was mutated in Arabidopsis. At the same time, double mutants (*dmr6dol1*) showed complete resistance to *H.arabidopsidis.* For activation of plant immunity, these mutants required SA accumulation. In Arabidopsis, DLO1, also called salicylic acid 3-hydroxylase, is encoded by a homolog of DMR6, which mediates leaf senescence and pathogen responses (Zhang *et al*., 2013). Disruption of DMR6 through the CRISPR/Cas9 system is a promising strategy to develop resistance against pathogens.

It is believed that knocking out of *DMR6* gene is a promising strategy to develop resistance against pathogens in crops as it shows resistance against many pathogens, including bacteria, fungi, and oomycetes. In the current study, *the DMR6* gene of *Nicotiana tabacum*was targeted with the CRISPR-Cas9 system. It is anticipated that engineered tobacco plants containing mutated *DMR6* gene helpful for resistance against pathogens such as *Pseudomonas* causing angular leaf spot disease of *Nicotiana tabacum.* For this purpose, gRNA oligos targeting *the DMR6* gene were synthesized commercially and ligated into plant expression vector pHSE-401. Then this construct was transformed into *E.coli*. The final pHSE401-gRNA-Cas9 construct was transformed into *Agrobacterium*. The T-DNA inserted this complex into the plant for insertional mutagenesis.

c. Improved Quality

Agriculture is the backbone of Pakistan's economy. Oilseed crops are of great concern in the agriculture sector. A wide range of oilseed plants has different fatty acid profiles due to different levels of fatty acids. Genetic variability in oilseed crops motivates researchers to search out the different potentials of oilseed crops and also improve their potential. Oilseed crops include soybeans, Sunflowers, cotton, linseed, olive, palm, coconut, and Brassica. These oilseed rape crops contribute a lot to the economics and health of people worldwide. In Agriculture, Brassica contributes a lot by providing several vegetables, a vast amount of edible oil, and industrial oil to the world. Brassica has provided the healthiest vegetable oil on the market: canola oil containing zero erucic acids (less than 1%), low Glucosinolates level, 60% oleic acid, 20% linoleic acid, and 10% α-linolenic acid.

The oil of *Brassica campestris* has a most suitable percentage of oleic acid, linoleic acid, and linolenic acid in it but has a higher concentration of erucic acid. The oil obtained from the Brassica is not only used for salad but also as cooking oil which is of good quality in the world. Brassica contains 3.5% α-linolenic acid by weight, 77% oleic acid by weight, Erucic acid is less than 2%, and total saturated fatty acids are not more than 4.5%. Oil quality has an inverse relation with protein content present in seeds of rapeseed. It means higher protein contents decrease the oil quality in Brassica. Cruciferous, napkins, and oleosins make protein fraction in the seed of Brassica. Genotype and environmental conditions also determine the oil quality in Brassicaceae.

Lycopene is an integral part of our diet. There are many experiments done to improve the concentration of lycopene in tomatoes. Lycopene is primarily present in large amounts in ripped tomatoes. Nevertheless, there is a feature of lycopene as it changes into beta-carotene due to lycopene beta-cyclase. So, we make two constructs called OE and AS. OE construct has high expression of the b-cyclase gene, while AS shows low expression. These constructs introduce in the tomato with the help of Agrobacterium-mediated transformation. In this experiment, we use the PDS promoter, as it helps to increase the expression. In the end, we conclude that AS has gene expression that shows a high amount of lycopene and has 50% less expression of B-cyclase. Bioactive compounds are ample nutritional constituents primarily found in small amounts in food. Lycopene is also a bioactive compound, so its proper amount is essential for health. CRISPR Cas9 method modulates genes that essential code enzymes that help metabolic pathways. Ultimately, its purpose is to enhance lycopene production and improve fruit quality (Čermák, Baltes, Čegan, Zhang, & Voytas, 2015) (R. Li et al., 2018; Nonaka, Arai, Takayama, Matsukura, & Ezura, 2017) (X. Li et al., 2018). Aluminum- activated-malate transporter 9 (ALMT9) is an integral part of malate content in tomatoes, and it is identified through CRISPR Cas9 (J. Ye et al., 2017). In tomatoes, the locule plays an indispensable role in the fruit size and contributes a maximum of 50% in the size enlargement of fruit. These locules are present in the flower petals. Through CRISPR Cas 9, we identified different quantitative trait loci that control the number of loci in fruit (Li et al., 2017). In the Cold Spring Harbor Laboratory, some scientist damages the typical stem cell system known as CLAVATA-WUSCHEL (CLU-WUS) and produces a large tomato fruit using CRISPR Cas9. (Ma et al., 2015) For the production of transgenic fruit more significant than the WT plant, eight sgRNA interacted with the CLV3 gene (promoter region) (T. Wang, Zhang, & Zhu, 2019). To increase the shelf life of tomatoes, ripening inhibitors and DNA demethylase 2 inactivates by using CRISPR (Lang et al., 2017) (Ito, Nishizawa-Yokoi, Endo, Mikami, & Toki, 2015).

Conclusion

Agriculture provides food (milk, meat, and eggs), medicines, wood, clothes, waxes, silk, honey, and many other valuable products. In short, agriculture plays a vital role in the survival of living organisms on earth. However, due to extreme climate changes, the production rate of plants is decreasing daily. In every era, scientists try to improve the situation. In this era, biotechnology is the hopeful solution for this significant problem. Different genome editing techniques can modify plant genomes to combat climatic changes and improve quality and yield. CRISPR/Cas is one of the best options to edit the genome in plants without significant side effects. Like in the case of abiotic and biotic stresses, CRISPR plays a significant role in combating these stresses. Also, it helps to enhance the nutritional quality of fruits and vegetables. Environmental challenges, as well as the backdrop of global climate change, the application of CRISPR to improve plants is crucial. To fully reap the CRISPR revolution's benefits, we should address its regulatory concerns.

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