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APPLICATIONS OF CRISPR-CAS9-MEDIATED GENE EDITING FOR CONTROLLING ABIOTIC STRESS IN CROP PLANTS.

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Abstract

Global agricultural production is significantly reduced by abiotic stressors such as drought, salinity, cold, heat, and heavy metals. To deal with these environmental pressures, techniques like transgenic and conventional breeding have been applied extensively. Because of its ease of use, accessibility, versatility, adaptability, and broad range of applications, the clustered regularly interspaced short palindromic repeat-Cas (CRISPR/Cas) based gene-editing technology has revolutionized. The development of crop varieties with improved resistance to abiotic stressors is a promising use of this technology. In order to improve tolerance to stresses like as drought, salinity, cold, heat, and heavy metals, we have applied the CRISPR/Cas mediated gene-editing system. Here, we provide an overview of the most recent research on this topic. Additionally, we emphasized in this review the most current developments in base and prime editing tools for crop enhancement.

Keywords: Abiotic stress tolerance, prime editing, CRISPR/Cas9, base editing, crop production, and gene editing

Introduction

Abiotic stressors that adversely impact agro-ecological conditions and agricultural output include drought, heat, cold, salt, and metals. These abiotic stressors are the main growth variables that, on a worldwide scale, restrict crop plant yield and quality. The plant experiences morphological, physiological, biochemical, and molecular alterations as a result of these abiotic influences, which reduces yield and production. By 2050, there will be 9.7 billion people on the planet, and in order to feed them all, agricultural productivity would need to increase by at least 85%. However, the traditional breeding strategy has made a significant contribution to crops' ability to withstand abiotic stress. To improve abiotic stress tolerance, however, traditional breeding is a valuable strategy for raising crop yield. On the other hand, it can take this approach years or even decades to build abiotic stress tolerance. To address these issues, more cutting-edge, effective solutions with immediate effects are undoubtedly needed. A technique for altering an organism's DNA by targeted

mutation, insertion/deletion (indel), and particular sequence alteration using specific nucleases is made possible by genome editing (also known as gene editing) tools. Meganucleases, zinc-finger nucleases (ZFNs), transcription activator-like nucleases (TALENs), and CRISPR-Cas9 have all been developed and employed for genome editing in recent years. The most sophisticated and effective method of editing the genome of a variety of organisms, including plants, is the clustered regularly interspaced short palindromic repeats (CRISPR)/CRISPR-associated protein 9 (CRISPR/Cas9) system.

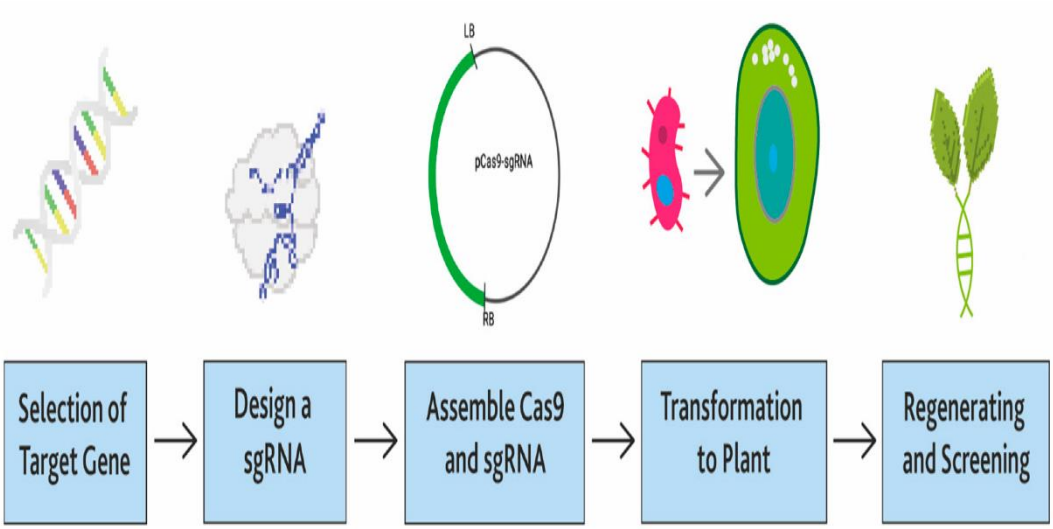

Figure 1: Recent Developments in CRISPR/Cas9 Genome-Editing Technology

Compared to other genome editing instruments, it is more accurate, quicker, less expensive, and more efficient. Furthermore, the genome's complementary DNA sequences can be found and broken using this technique. It was modified from a bacterial gene-editing mechanism that exists naturally to offer defense against invasive viruses. It is currently acknowledged, nevertheless, as a component of an adaptive defense mechanism that also involves CAS enzymes connected to CRISPR/Cas9. Because of the "foreign DNA" approach, this technique may further facilitate and encourage the use of CRISPR/Cas9-based products to overcome challenges with cultural acceptance. Few nations, nevertheless, have embraced genome-edited crops; in contrast, a number of other nations are still discussing the issue. The Japanese market has recently seen the commercialization of CRISPR/Cas9 modified tomatoes, which have higher levels of γ-aminobutyric acid (GABA) than their non-edited counterparts. It is anticipated that this technology would significantly contribute to most countries' relatively easy acceptance of genome-edited crops [1-5].

Numerous plant species, including model plants like Nicotiana benthamiana,

Nicotiana tabacum, and Arabidopsis, as well as crop plants including wheat, maize, rice liverwort, tomato, potato, soybean, sweet orange, banana, pepper, and sugar cane, have successfully used the CRISPR/Cas9 system. Furthermore, CRISPR-Cas9-based multiplexing, which targets many genes in a single organism, has been effectively applied to a variety of crops, including maize, wheat, rice, and cotton. Thus, by simultaneously targeting multiple stress-sensitive genes in an elite, high-yielding, but sensitive cultivar, this technology has enormous potential to produce genome-edited crop plants tolerant to multiple stresses. Tolerance genes can also be overexpressed using CRISPR-mediated gene activation. According to Mushtaq et al., the CRISPR/Cas-based gene-editing technique might effectively target complicated quantitative genes that are either directly or indirectly linked to abiotic stressors. Using CRISPR/Cas9 mediated gene editing, it was discovered that a number of rice genes, including betaine aldehyde dehydrogenase (OsBADH2), mitogen-activated protein kinase (OsMPK2), stress/ABA-activated protein kinase 2 (SAPK2), and phytoene desaturase (OsPDS), were involved in abiotic stress tolerance. Through gene editing of the transcriptional factor ethylene responsible factor (ERF) of the AP2/ERF superfamily, abiotic stress tolerance in plants was demonstrated [6-9].

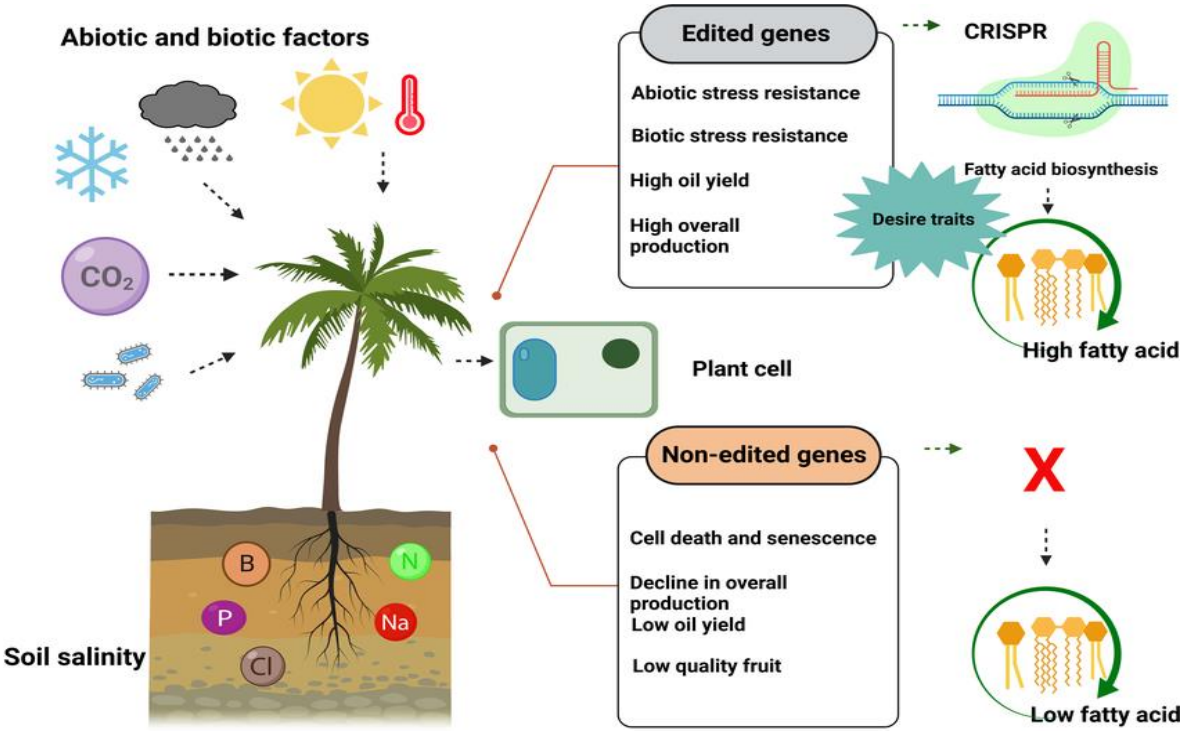

Figure 2: CRISPR/Cas9-mediated genome editing of palm trees

At the moment, CRISPR/Cas-based genome engineering is effectively being utilized to understand plant tolerance to a variety of abiotic stimuli, such as heat, salt, drought, and nutritional values in a number of significant crop species. In this review, we address the future potential of this technique for the production of stress-tolerant crop varieties and highlight the majority of possible uses of the CRISPR/Cas9 driven genome editing strategy in crop plants for regulating abiotic stresses such heat, salinity, drought, and other environmental conditions [10].

Mechanistic overview of genome editing method based on CRISPR-Cas9

The adaptive immune system found in bacterial and archaeal genomes to defend against the invasion of foreign plasmids or viral DNA is the foundation of the CRISPR/Cas system. A single guide RNA (sgRNA) and the CRISPR-associated protein 9 (Cas9) are important components of the CRISPR/Cas9 system. The transactivating crRNA and the protospacer-matching CRISPR RNA (crRNA), which are required for CRISPR activity, are combined synthetically to form the sgRNA. The twenty nucleotides at the 5' end of a sgRNA are part of the Cas9/sgRNA complex, which attaches to the desired location in the target genome. This particular target site needs to be situated right upstream of the protospacer adjacent motif (PAM; NGG for SpCas9 from Streptococcus pyogenes). This motif is a short conserved DNA sequence that is downstream of the cleavage site and varies in length depending on the species of bacteria. Its length usually ranges from two to five base pairs. Known as a genetic scissor, the SpCas9 protein is a large (1368 amino acids) multi-domain DNA endonuclease that cleaves target DNA in the genome to form a blunt-ended double-strand break (DSB). Ultimately, the host cellular machinery fixes the DSB. The two processes that repair the double-stranded breaks created by the Cas-9 protein are non-homologous end joining (NHEJ) mechanisms and homology-directed repair (HDR). Homology-directed repair uses a homologous DNA template and is incredibly accurate. HDR requires a lot of donor DNA templates that include a target DNA sequence because it is mostly active in the late S and G2 phases of the cell cycle. The precise gene insertion or replacement is carried out by appending a donor DNA template exhibiting sequence homology at the anticipated double-strand break location. Non-homologous end-joining expedites the repair of double-strand breaks (DSBs) by enzymatically combining DNA fragments without the need for exogenous homologous DNA. NHEJ is a constitutive repair mechanism primarily active in cells that is typically active in all phases of the cell cycle. NHEJ causes somatic cells and typically produces mutated products with short base insertions

or deletions (indels), which causes the targeted genes to become changed. But because of its vulnerability, it may result in small, random insertions or deletions (indels) near the cleavage site, which could produce premature stop codons or frameshift mutations. Figure 1 provides a schematic representation of the mechanistic insights of CRISPR-Cas9-based genome editing in plants [11-15].

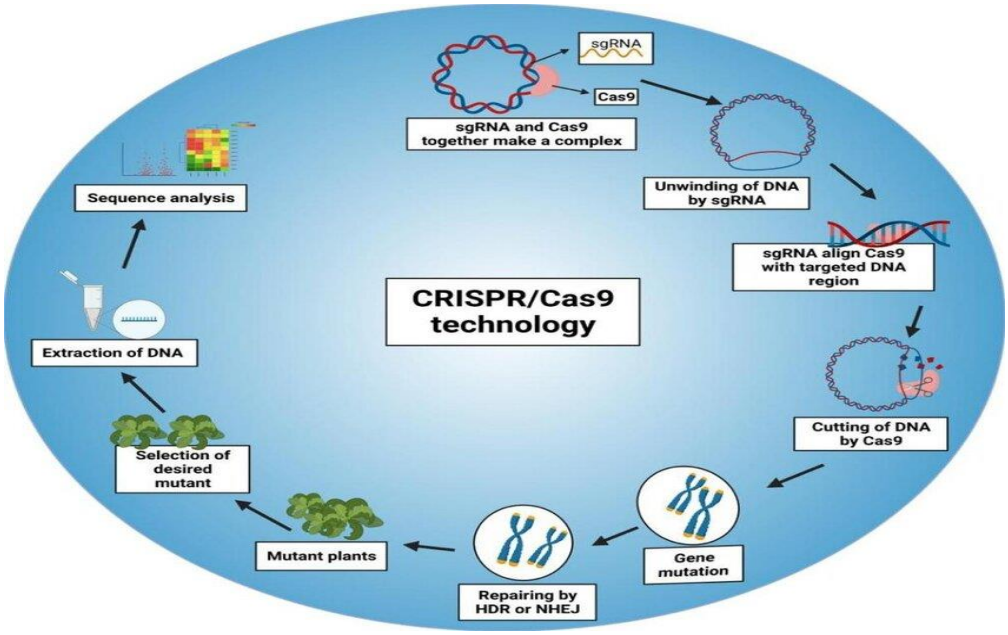

Figure 1. Diagrammatic representation of the mechanistic insights of plant genome editing using CRISPR-Cas9. A chosen single guide RNA (sgRNA) directs the Cas9 protein, which then causes a double-strand break (DSB). Then, non-homologous end-joining (NHEJ) or homology-directed repair (HDR) mechanisms take over and repair DNA. BioRender.com (https://app.bioren- der.com/biorender-templates) was used to make the figure, which was accessed on May 25, 2022.

Innovative technical methods and tactical recommendations for genome editing

New avenues for the functional investigation of genes are opened by novel gene editing techniques derived from the CRISPRs-Cas9, base editing (BE), and prime editing (PE) technologies. Cas9's editing efficiency may be increased by skillfully vetting desired traits, examining genetic material by gene knockout, and employing a final genetic transformation process. Here, we report a revolutionary development that will improve agricultural plant genome editing. The creation of mutant libraries, transgene-free genome editing, base and prime editing, and multiplexed CRISPR technologies for gene editing have all been enumerated as methodologies.

Prime editing and base editing

A single base alteration in the gene can improve a number of significant crop attributes; donor DNA templates and DNA DSB events are not necessary for HDR. In these circumstances, single base editing cannot be achieved using the knock-in/out strategy in the standard CRISPR-Cas system. Agronomic trait reports have shown that many of these qualities are determined by variations in the single bases of genes [46]. Unfortunately, due to its limitations, gene base conversion cannot be performed using the CRISPR/Cas9 method. As a result, it is best suited for genes that are either knocked out or added to the genome. Finding a reliable and consistent technique to modify crop plants' genomes is essential in light of these limitations.

Base editing is a cutting-edge editing technique that fulfills this need and is thought to be a more effective and efficient tool than its predecessor [47]. It's an easy-to-use and precise technique for changing nucleotides without causing DNA double strand breaks [48, 49]. The gRNA-guided target discovery in the genome is still necessary for the base editing pipeline, but it also involves the inactive CRISPR–Cas9 nuclease, unable to create double-strand breaks, coupled to the adenosine or cytosine deaminase enzyme component, which modifies nucleotide conversion. The transition base shifts from C to T and A to G are catalyzed by the cytosine base editors (CBEs) and adenine base editors (ABEs), respectively. Different CBES and ABE generations have developed after their initial use and later for sophisticated applications with higher editing efficiency. One base edit can be made at a time by ABEs and CBEs. The dual base editor, which can produce both C to T and A to T base replacement, was therefore created by combining both editing processes onto a single platform, thereby increasing the possibilities of base editing.

Additionally, attempts are being undertaken to develop base editors for transversion base substitution in order to expand the usefulness of base editing beyond merely limiting it to base editing with transition base conversion. A Cas9 nickase (nCas9-D10A) is fused to a cytidine deaminase and a uracil-N-glycosylase (UNG) to form C-to-G Base Editors (CGBE). A useful alternative is suggested by a recently developed cytidine base-editing program [50]. Acetolactate synthase (ALS), a gene involved in wheat production, is a prime candidate for base editing to give herbicide tolerance. Point mutations within ALS can confer sufficient herbicide tolerance while having little effects on plant productivity. For this reason, cytidine base editing has been used to create mutations in ALS genes in a few diploid plant species,

such as Arabidopsis' tribenuron tolerance and rice's imidazolinone tolerance.

The flexible and precise gene-editing technique known as prime editing (PE) makes use of nCas9 joined to a specially designed reverse transcriptase. In contrast to HDR-dependent CRISPR-Cas9, it is often referred to as homology-directed repair (HDR)-independent CRISPR-Cas9. Template RNA is simultaneously coupled to sgRNA to create unique prime editing guidance RNA (pegRNA), which specifies the target site and encodes the expected editing sequence. It has been reported that pairs of pegRNA can precisely replace a 108 bps sequence or delete 710 bps. Prime editing has been used on tomatoes, rice, corn, and wheat thus far [16-19].

Editing Transgene-Free Genomes

Across plant species, gene editing is widely employed to investigate and generate the effects of functional mutations in crop improvement. On the other hand, the incorporation of transgenes into plant genomes presents important legal questions about genetically modified species. In order to encode changed DNA sequences into the host genome, traditional genome engineering techniques require the transfer and mixing of DNA cassettes. Although DNA fragments are usually degraded, they nevertheless have negative consequences. Because of its benefits, DNA-free genome editing is emerging as a novel and quickly growing field in the biological sciences. This method improved the prospects for creating organisms that are not genetically modified and made it possible to make precise genome modifications without interfering with the genome. Nevertheless, the same fundamental problems that plague transformation techniques also affect genome editing without DNA. Plant cells lack the same level of development in RNA and protein transport strategies as animal cells, despite the latter having access to a wider range of transfer methods. Transgene-free genome-edited plants could therefore only be produced by protoplast transfection and the biolistic approach [62]. The first tissue to be effectively targeted for DNA-free gene editing using polyethylene glycol (PEG) mediated fusion was protoplasts. As a result, the protoplast and the ribonucleoprotein (RNP) complex or mRNA merge with PEG.

By transfecting guide RNA and the Cas9 protein into the protoplasts of rice, lettuce, tobacco, and Arabidopsis thaliana, researchers were able to study DNA-free genome editing and achieve targeted mutagenesis in regenerated plants at frequency of up to 46%. Using CRISPR/Cas9 RNAs, particle bombardment of the wheat embryo has been utilized to create genome-edited plants without DNA.Through a transient expression method, our team

accomplished very accurate and efficient DNA-free genome editing, resulting in homozygous mutant plants in the T0 generation. Furthermore, using circular and linearized plasmid DNA fragments, a recently published study by demonstrated an effective method of DNA-free genome editing in potato (Solanum tuberosum) protoplasts, demonstrating high transgene expression and up to 95% gene editing events in potato calli derived from protoplasts.

Gene editing using multiplexed CRISPR technology

Multiplex genome-editing technologies are flexible and effective methods for fine-tuning many distinct DNA loci within the genome. This method considerably increases the possibilities of genome editing efficiency and facilitates powerful bioengineering applications by simultaneously expressing several gRNA and Cas9 enzymes. The attainability of targeted modifications at several nucleotide levels in the target genome has been greatly enhanced by these methods. Any crop plant can have many genomes altered at once by using multiple sgRNA targets. This method could be used to introduce new plant kinds and various features. Moreover, a plasmid vector containing many sgRNAs might be used to target a large number of individuals from various families. Multiplex genome editing, which is widely utilized to edit multiple sgRNA targets in the genome, is the main benefit of CRISPR. It is done in two ways: in the first, many sgRNAs are directed to express as a single transcript using a single promoter. In a different instance, every sgRNA target is expressed via a single promoter. Multiplex genetic engineering mostly focuses on features such as herbicide tolerance in plants. However, it has since been expanded to include other aspects, including hormone biosynthesis and perception with over 100 simultaneous targeting events, metabolic engineering, plant development, and molecular farming. Targeting multiple closely related sequences at once, such as multiple alleles of the same gene, members of the same gene family, or homoalleles in polyploid plants, is known as multiplex genome editing. Thus, the ability to modify many genomes will provide a faster means of creating novel crop kinds that are economically significant [20-21].

Creation of Mutant Library Collections

The high-throughput mutagenesis strategy can be improved due to the great efficiency of CRISPR-mediated mutagenesis of agricultural plants. The widespread application of CRISPR/Cas9 is crucial for creating mutant libraries and understanding the genetic mechanisms underlying crop improvement. Making mutant libraries is a useful and effective technique. The 18–20 bp target binding order of the sgRNA target can be changed to modify the tar-geting capabilities of CRISPR/Cas9, a powerful tool for creating mutant libraries. To

create a set of mutant lines with the fewest transformation tries and in the shortest amount of time, pooled CRISPR libraries were transformed for the purpose of altering tomatoes. In this work, immunity-related leucine-rich repeat subfamily XII genes were the subject of a single transformation attempt utilizing the CRISPR library, which led to the retrieval of inherited mutations in 15 out of the 54 targeted genes. They also created a second library with three sgRNAs per construct to target 18 genes, which led to mutagenesis in 15 of the 18 targeted genes overall in an effort to increase productivity. Mutant libraries including loss-of-function mutations were created for the rice plant transformation. During field cultivation, these plants displayed phenotypic alterations such as sterility and lethality.

Effects of CRISPR-Cas9-mediated gene editing on stress tolerance and plant yield

Abiotic stress has a detrimental effect on the growth and yield of plants. It has an impact on several physiological, morphological, and biochemical factors that are essential to plant growth. Currently, about 20 agriculturally important crops have embraced CRISPR-Cas mediated gene editing. After ABA-induced transcription repressors (AITRs) were discovered to be a novel family of transcription factors, ABA sensitivity in Arabidopsis decreased as a result of the loss-of-function of AITR genes, which are crucial feedback regulators of ABA signaling. Arabidopsis was able to withstand abiotic stresses, such as salt and dehydration, thanks to changes in AITR gene expression.

On the other hand, Arabidopsis with AITR5 gene overexpression displayed a worse tolerance to salinity stress. Notably, Arabidopsis with six AITR genes knocked out exhibited enhanced resistance to salinity and drought, even when fitness cost was considered. Similar to this, the Arabidopsis plant with triple mutant showed tolerance to drought and salt stress when three AITR genes—aitr2, aitr5, and aitr6 (aitr256)—were knocked out using CRISPR/Cas9 technology. This resulted in quintuple mutants, increasing the plants' potential to withstand stress. The rice plant exhibited enhanced resistance to abiotic stressors when OsmiR535 and a zinc finger transcription factor were knocked down using CRISPR/Cas9. These mutant plants also displayed improved root, shoot, and leaf architecture, as well as increased leaf water retention and stomatal conductance. To build stress tolerance in plants, CRISPR-Cas9-based editing of abiotic stresses, such as heat, cold, salt, drought, and heavy metal stress-responsive genes and their negative regulators, as well as plant transformation techniques

Stress tolerance during a drought

Increased accumulation of metabolic and signalling molecules as well as improved drought tolerance in plants are caused by the overexpression of a number of transcription

factors and genes that are responsive to drought. Plants that express drought-sensitive (S) genes are more vulnerable to drought because of hormonal imbalances, decreased antioxidant activity, and increased formation of reactive oxygen species (ROS). While the AREB1 knock-out mutant displayed increased vulnerability to drought stress, over-expression of AREB1 has demonstrated enhanced tolerance to drought stress. When compared to overexpressing transgenic and wild-type tomato plants, CRISPR/Cas9 induced the mutation of SlLBD40, a transcription factor located in the lateral organ boundaries domain that improves drought tolerance in tomatoes. Moreover, the CRISPR/Cas9 deletion of SlLBD40 improved tomato drought tolerance. Tomato (Solanum lycopersicum) mutant plants using CRISPR-Cas9 editing that had the SlMAPK3 gene knocked out demonstrated an improved sensitivity to drought stress. These mutant plants showed signs of severe wilting, increased H2O2 levels, decreased antioxidant levels, and enhanced membrane damage during drought. These findings support the hypothesis that SlMAPK3 protects the cell membrane and plays a role in tomato plants' response to drought stress. Tomato tolerance to water deprivation is enhanced by the knockout of the tomato Auxin Response Factor (SlARF4) gene. Arabidopsis showed enhanced resilience to drought stress through CRISPR/dCas9 fusion with the Histone Acetyl Transferase (AtHAT) gene. Through increased stomatal aperture, malondialdehyde (MDA) level, H2O2 content, and ion leakage, CRISPR-Cas9-based editing of the pathogenesis-related 1 (NPR1) gene in tomatoes demonstrated drought responsiveness. Nevertheless, it was discovered that the antioxidant activity level was lower than in WT plants. In tomatoes and other crop plants, the SlNPR1 is crucial in guiding responses against drought stress. Gene editing can be used to develop several SlNPR1 variations that improve drought tolerance over a broad range [39].Negative regulators of drought tolerance include the genes for drought-induced SINA protein 1 (OsDIS1), drought and salt-tolerant protein 1 (OsDST), and ring finger protein 1 (OsSRFP1).

These genes that respond to drought enhanced antioxidant enzyme levels, reduced H2O2 concentrations, and strengthened rice plants' resistance to drought stress. Plants' responses to dehydration and ABA signaling are regulated by the enhanced response 1 (ERA1) protein gene. OsERA1 gene genome editing improved rice's ability to respond to drought stress. Under drought conditions, the mutant plant displayed enhanced sensitivity to ABA and stomatal closure. The significance of OsSAPK2 in ABA-mediated stress tolerance in rice was also established by creating mutants with loss of function mutations using CRISPR-Cas9. In comparison to WT plants, the mutants developed showed increased sensitivity to drought. The

CRISPR Cas9-induced mutations in the gene encoding OPEN STOMATA 2 (AtOST2) in Arabidopsis mutants compared to WT allowed an enhanced stomatal response. Interestingly, there was a significant level of stomatal closure in the AtOST2 mutants. The OsSRL1 and OsSRL2 genes in rice encode the phenotypic of leaf tissue. In comparison to the wild-type one, the genome-modified lines with homozygous SRL1 and SRL2 mutant were shown to exhibit retardation in a variety of characteristics, including stomata number, stomatal conductance, transpiration rate, chlorophyll content, vascular bundles, and other agronomic properties. By tar-geting negative regulators or drought-sensitive genes, CRISPR-Cas9 based genome editing can confer drought resistance. In order to establish drought tolerance, CRISPR-Cas9-based gene editing was used to increase the expression level of the ARGOS8 gene, which adversely affects the ethylene response. In the field, these mutant plants produced more grain when subjected to drought stress. The growth and development of plants, as well as their response to biotic and abiotic stressors, are regulated by WRKY transcription factors. The regulation of defense responses in plants to drought stress is significantly influenced by the WRKY3 and WRKY4 genes.

Benefits of genome editing over transgenic and breeding methods

Crossbreeding is the traditional breeding method used to transfer beneficial features from a donor line to superior recipient lines. In a similar vein, random mutations are introduced genome-wide by mutation breeding, which significantly increases genetic diversity. But in order to guarantee that only the desired trait is transferred, repeated backcrossing to the recipient's background must be done, which takes a long time (8–12 years). Transgenic breeding can also be used to transfer useful genes or features from other animals, however this method entails the random integration of foreign DNA into the genome. Therefore, before being commercialized, any transgenic line creation will need to go through the drawn-out and expensive regulatory evaluation process. Of all these techniques, genome-editing technology offers the potential benefit. Plant DNA can be precisely altered through genome editing to resemble potential natural alterations. It does not require the integration of foreign DNA and can effectively edit the plant genome for the improvement of traits. In this situation, repeated backcrossing is not necessary, and transgene-free lines can be available in as little as two to five years. Therefore, vital qualities like increased resistance to temperature extremes could become available more quickly, assisting in the assurance of a stable food supply. Many nations, including the USA, China, India, UK, and many more, have recently permitted genome-edited plants to go through regulatory procedures apart from those

governing genetically modified goods. The ability to multiplex, or edit several target sites at once, is one of the benefits of CRISPR tools over other genome-editing technologies. The most significant benefit of gene editing over earlier technologies is its ability to modify a single gene specifically and across the entire plant genome. With the use of modern plant breeding techniques that utilize CRISPR technology, it is possible to transmit the desired feature to progeny that lack efficacy through a simpler, more accurate, and versatile form of mutagenesis. Because this process may be automated to target specific regions of genetic code or change DNA with more accuracy, the properties of the plants become even more significant. It can execute mutations to an exact place inside the targeted gene [22].

Final thoughts and prospective directions

Since CRISPR/Cas9 is very efficient, inexpensive, and simple to use, it is regarded as the preferred way of genome editing compared to other genome editing techniques like ZFNs and TALENs. Sequence-specific editing has been used to change a variety of plant species in order to characterize the function of genes and ultimately exploit them for trait improvement. By using many gRNAs, it can cause editing at several different locations throughout the genome. This is useful for targeting numerous members of multiple gene families and stacking features in an elite variety. This approach makes advantage of site-specific modifications to the genome, including base editing, epigenetic modifications, and gene expression regulation. The effector protein of dead Cas9 (dCas9) protein, which is catalytically dead but still has DNA binding activity, is fused to accomplish this. In this manner, the fusion protein is directed to operate at particular locations throughout the genome. Without editing, the CRISPR interfering system (CRISPRi) may be able to generate accurate and efficient transcriptional regulation. Thus, this technology is superior to RNAi technology. This occurs once more when SgRNA binds to dCas9. When the complementary region of the SgRNA binds to it, RNA polymerase is prevented from elongating transcription, allowing the gene to be expressed without causing cell death or genomic damage. The CRISPR/Cas system can also be used to replace genes in plants by utilizing homology-mediated recombination to target and integrate particular genes. Furthermore, in a heterozygous system, the CRISPR/Cas system can reassemble the genome following the DSB. This can be used to narrow down advantageous QTL to the causal allele for accurate mapping and gene identification. It can also be used to induce local recombination in the regions of the chromosome that do not participate in meiotic recombination, such as the telomeric end and centromeric region.

Its efficiency and target specificity have improved significantly, but more interventions are needed to make it an even more potent instrument. Introducing the more compact CRISPR system for effective genome editing is one of the few such domains. Since it is relatively big, the current CRISPR/Cas9 cannot fit into viral vectors. In a similar vein, not all regions of the genome can be edited by CRISPR due to the requirement of the NGG PAM site. Consequently, choosing more than one PAM site will broaden the editing's scope. To create transgenic events, the transformation rate and editing efficiency with agrobacterium-based techniques are favored; yet, not all plant species, including crops, react favorably to the transformation and regeneration under selection. Furthermore, it takes longer to remove the transgene by multiple backcrossings of the edited plant in order to make it transgene-free. RNP, viral delivery, and nanoparticle-mediated delivery are tissue culture-free techniques that offer different approaches to quicken the procedure. The process of creating an altered plant will be simpler, less expensive, and need less time. RNPs are regarded as transgene-free since they carry out the editing without leaving any trace in the genome. The plasmid may now be delivered via nanoparticles, and work is being done to load and distribute the RNPs. While HDR is an uncommon occurrence following a DSB in the genome, NHEJ repair happens often, and HDR is necessary for a number of purposes, including recombining the genome. Consequently, any protein that improves HDR should be sought out and directed to DSB sites where it can fuse with CAS protein [20-22].

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