Review

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Genetic Databases and Gene Editing Tools for Enhancing Crop Resistance against Abiotic Stress

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Abstract: Abiotic stresses extensively reduce agricultural crop production globally. Traditional breeding approaches have been widely used to mitigate the risks of abiotic stresses. The discovery of gene editing technology for modifying stress-responsive genes and associated molecular networks has paved the foundation for sustainable crop management against environmental stress. Integrated approaches based on functional genomics and transcriptomics are now expanding the opportunities to elucidate the molecular mechanisms underlying abiotic stress responses. This review summarizes some of the features and weblinks of plant genome databases related to abiotic stress genes utilized for crop improvement. Clustered, regularly interspaced short palindromic repeats (CRISPR)/CRISPR-associated protein 9 (Cas) based gene-editing tool has revolutionized stress tolerance research due to its simplicity, versatility, adaptability, flexibility, and broader applications. However, off-target and low cleavage efficiency hinder the successful application of CRISPR/Cas systems. Computational tools have been developed for designing highly competent gRNA with better cleavage efficiency. This powerful genome editing tool offers tremendous crop improvement opportunities, overcoming conventional breeding techniques' shortcomings. Furthermore, we also discuss the mechanistic insights of the CRISPR/Cas9-based genome editing technology. This review summarizes the current advances in understanding plant species' abiotic stress response mechanism and applying the CRISPR/Cas system genome editing technology to develop crop resilience against drought, salinity, temperature, heavy metals, and herbicides.

Keywords: abiotic stress; CRISPR/Cas9; genome editing; genome databases

1. Introduction

Agricultural production faces global challenges due to climate change, insufficient arable land, population growth, and abiotic and biotic stresses. Abiotic stress adversely impacts plant growth and development by hampering essential biochemical and physiological activities [1,2]. Climate change, such as extreme temperature, drought, water logging, flooding, and increased soil salinity, leads to various abiotic stresses threatening agricultural food production worldwide. Excessive greenhouse gas emissions are responsible for frequent high temperatures and drought stress in crop plants. Drought stress impacts crop plants, including alterations in growth patterns, plant physiology, genotypes, developmental stages, growth durations, and defense mechanisms [3–5]. Salinity stress damages biomolecules, including nucleic acids, proteins, and lipids, by generating reactive oxygen species (ROS) [6,7]. Plants respond to heat stress in various ways, including alterations in enzymes that generate reactive oxygen species (ROS), heat shock proteins (HSPs), and genes encoding scavenger proteins [8,9]. Temperature stress strongly impacts grain filling, leading to poor grain yield [5,10]. In addition, overusing chemical fertilizers/herbicides contaminates agricultural fields with

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heavy metals such as arsenic (As), copper (Cu), cobalt (Co), cadmium (Cd), iron (Fe), manganese (Mn), nickel (Ni) zinc (Zn), mercury (Hg), lead (Pb) accumulating inside plants, reducing grain fullness, and increasing risk to human and animal health. Arsenic causes a loss of functionality in plant cells, and cadmium inhibits plant growth, as evidenced by stunted plant growth, a decrease in leaf size, shoot growth, and root dry matter [11–14]. Plants evolve several defense mechanisms to withstand stress by activating stress-responsive genes via secondary messengers and finally activating several stress-responsive genes and their products [15].

Conventional breeding approaches, including cross-breeding and mutation breeding, have enhanced crop performance under climate change scenarios. However, even with marker-assisted selection, breeding programs can be time-consuming and labor-intensive [16]. Therefore, additional efficient and cutting-edge technologies with immediate impacts are required to overcome the drawbacks of traditional breeding methods. The availability of genome sequence data of numerous crop plants and precise genome editing tools has revolutionized plant breeding programs. Genome editing tools enable desired changes in an organism's DNA by introducing an insertion/deletion (indel) and mutation in the sequences of particular genes via recruiting specific nucleases such as zinc-finger nucleases (ZFNs) and transcription activator-like effector nucleases (TALENs) or Clustered regularly interspaced palindromic repeats (CRISPR)/ CRISPR-associated protein 9 (Cas9) system [17]. CRISPR/Cas comprises single-guide RNA (sgRNA) and RNA-guided Cas endonuclease that protects bacteria and archaea from being invaded by mobile genetic elements and bacteriophages [18]. During the process of genome editing, sgRNA recruits Cas endonuclease to a specific site of the genome to catalyze a DNA double-stranded break (DSB), which can be repaired by various DNA repair mechanisms, including non-homologous end joining (NHEJ), homology-directed repairs (HDR), and microhomology-mediated end joining [MMEJ], leading to gene knockout, DNA fragment insertion, deletion, and replacement [19–22]. Different Cas enzymes recognize Protospacer-Adjacent Motif (PAM) sequences and show unique specificity. For example, SpCas9 orthologues have been identified from *Streptococcus canis* (ScCas9), *Staphylococcus aureus* (SaCas9), *Streptococcus thermophiles* (St1Cas9), and *Brevibacillus laterosporus* (BlatCas9) to edit plant genomic loci having PAM sequence of NNG, NNGRRT, NNAGAAW, and NNNCND, respectively [23]. Moreover, the gRNA spacer sequence could be readily programmed using online tools to target DNA sites with PAM. Therefore, a guide sequence that matches or is highly similar to multiple genomic loci can be avoided to prevent off-target effects that may lead to undesired genetic modification in the genome of the cell or organism [24].

CRISPR technology has revolutionized life science research since it was first applied in 2012. CRISPR–Cas9 and CRISPR–Cpf1 are plants' best-studied and most widely used CRISPR systems [25–27]. In addition, Cas12a and Cas12b systems were also developed for plant genome editing [28]. CRISPR/Cas9 system has been applied to several plant species, such as *Nicotiana benthamiana* [29], *Nicotiana tabacum* [30], *Arabidopsis thaliana* [31], *Zea mays* [32], *Oryza sativa* [33], *Triticum aestivum* [34], *Hordeum vulgare* [35], *Setaria italica* [36], *Lycopersicon esculentum* [37], *Solanum tuberosum* [38], *Capsicum annuum* [39], *Brassica napus* [40] *Glycine max* [41], and *Saccharum spp.* [42]. This review summarizes plant genome databases related to abiotic stress and the potential applications of the CRISPR/Cas9-mediated genome editing approach in managing abiotic stresses such as drought, salinity, temperature, environmental pollutants, and heavy metal toxicity in important agricultural crop species.

2. Genome Databases of Abiotic Stress Gene

Genome databases dedicated to plant abiotic stress genes and genome data provide helpful information on essential plant species. The publicly available specialized bioinformatics database resources contain valuable information on plant stress genes, such as PlantStress, Plant Stress Gene, Plant Stress Proteome (PlantPReS), Stress Responsive Transcription Factor (STIFDB v.2), Plant miRNA ENcyclopedia (PmiREN), Network-based Rice Expression Analysis (NetREx), PncStress, and Pearl Millet Drought Transcriptome (PMDTDb) databases.

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2.1. PlantStress

Plantstress (https://plantstress.com/) website was launched in the year 2000 to serve as a web-based resource of information, a meeting place, a consultation facility, and a source for professional updates on the most critical issues of plant environmental abiotic stress, including drought, salinity, heat, mineral deficiency, oxidative, cold, water logging and stress combination [43].

2.2. Plant Stress Gene Database

The Plant Stress Gene Database (http://ccbb.jnu.ac.in/stressgenes/, accessed on 23 August 2023) contains 259 genes from 11 plant species involved in stress conditions such as drought, salinity, or heat. Moreover, it is also possible to obtain information about paralog or ortholog proteins coded by stress-related genes. Species present in the database are *Arabidopsis*, *Arachis hypogaea*, *Glycine max*, *Hordeum vulgare*, *Oryza sativa*, *Pennisetum glaucum*, *Phaseolus vulgaris*, *Saccharum officinarum*, *Lycopersicon esculentum*, *Triticum aestivum*, and *Zea mays* [44].

2.3. Plant Stress Proteome Database (PlantPReS)

PlantPReS (http://www.proteome.ir/, accessed on 23 August 2023) comprises more than 20,413 entries from 456 manually curated articles and more than 10,600 unique stress-responsive proteins [45]. It is an open online proteomic database comprising > 35086 entries from 577 manually curated articles containing>10600 unique stress-responsive proteins. A customized BLAST tool has been made available, which is helpful in retrieving the homologous sequences from the database. PlantPReS represents a precious resource for the plant stress community due to its user-friendly interface and several analysis tools, such as a search engine, gene ontology, cross-referencing, and expression patterns of target proteins involved in a stress response.

2.4. Stress Responsive Transcription Factor Database (STIFDB v.2)

The Stress Responsive Transcription Factor Database (STIFDB v.2) (https://ngdc.cncb.ac.cn/databasecommons/database/id/4666, accessed on 23 August 2023) plants stress-responsive transcription factor database containing information on stress-responsive transcription factor binding sites and stress-responsive genes in *A. thaliana* and *O. sativa subsp. japonica* and *subsp. Indica.* STIFDB contains more than 38,798 associations of stress-responsive genes and transcription factor binding sites [46]. The user can search the resource by chromosome, gene name, stress signal, and transcription factor.

2.5. Plant miRNA ENcyclopedia (PmiREN)

PmiREN (Plant miRNA Encyclopedia) is a functional plant miRNA database available at https://www.pmiren.com/, accessed on 23 August 2023. PmiREN contains 38,186 miRNA loci belonging to 7,838 families, 141,327 predicted miRNA-target pairs, and phylogenetic trees of conserved miRNA families in 179 species spanning from chlorophytes to angiosperms. It also provides tools for in-depth data mining. Additionally, 116 PARE-Seq libraries were utilized to confirm predicted miRNA-target pairs, and 2,331 fully sequenced small RNA libraries were used to quantify miRNA expression patterns [47].

2.6. Network-based Rice Expression Analysis (NetREx)

Network-based Rice Expression Analysis server (NetREx) provides information on the expression and interaction of rice genes under various environmental stress and hormonal treatment conditions. NetREx is a web-based server available at https://bioinf.iiit.ac.in/netrex/index.html, accessed on 23 August 2023. It offers a range of interactable data viewers and modules for analyzing user-queried genes across different stress conditions (drought, flood, cold, and osmosis) and hormonal treatments (abscisic and jasmonic acid). The server can also explore the expression fold change, gene annotations, and analysis of their nearby genes and linked pathways by querying

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subnetworks of user-defined genes in pre-built tissue-specific networks. This web server also allows the search of orthologous genes from *A. thaliana* , *T. aestivum, Z. mays, H. vulgare,* and *S. bicolor* [48].

2.7. PncStress

PncStress (https://bis.zju.edu.cn/pncstress/, accessed on 23 August 2023) is a manually curated archive of experimentally validated microRNAs, lncRNAs, and circRNAs responding to plants' abiotic and biotic stress conditions. It provides biological information and network visualization. PncStress possesses 4227 entries, including 2523 miRNAs, 444 lncRNAs, and 52 circRNAs validated by various experimental methods, with 114 species responding to 48 abiotic and 91 biotic stresses [49].

2.8. Pearl Millet Drought Transcriptome Database (PMDTDb)

PMDTDb (Pearl Millet Drought Transcriptome Database) is the database of drought transcriptome of pearl millet available at http://webtom.cabgrid.res.in/pmdtdb/, accessed on 23 August 2023. It catalogs the differentially expressed genes in leaf and root tissue of millet in response to drought along with transcription factors (TFs), gene regulatory network (GRN) having hub genes, and genic region putative marker discovery; simple sequence repeats (SSRs), single nucleotide polymorphism (SNP) and, InDels (insertions and deletions). This database is based on a 3-tier architecture developed in PHP and MySQL [50].

3. Functional Genomic Approaches and Abiotic Stress Tolerance

Functional genomics approaches have been employed to understand the precise regulatory gene networks associated with complex abiotic stress responses, benefiting crop improvement programs. Several stress-related genes/pathways and regulatory networks have been worked out in the past decades using various functional genomic approaches, including expressed sequence tags (ESTs), transcriptome analysis, and targeted random mutagenesis. Recent advancements in sequencing technology have provided a rapid and cost-effective method for generating enormous sequence data, facilitating the identification of genes/transcription factors mediating stress tolerance. Transcriptome analysis using microarray technology is a powerful technique that has proven helpful in identifying many stress-inducible genes. The identified genes can be successfully used in crop improvement programs by employing a transgenic approach or exploiting genetic variation associated with the trait of interest. Additionally, functional validation of stress-related genes may facilitate unraveling the stress-tolerance networks and designing different functional markers for crop improvement programs.

3.1. Sequencing-Based Approaches

Expressed Sequence Tags (ESTs) are the partial gene coding sequences generated by single-pass sequencing of cDNA clones [51]. EST databases are efficient tools for discovering genes, comparing interspecies sequences, and providing markers for physical and genetic mapping and clones for expression analysis. Functional genomics studies utilize ESTs due to their quick and cost-effectiveness compared to the whole genome sequencing method. Currently, over a million ESTs of different crop species are available in the EST database at the National Center for Biotechnological Information (NCBI) (http://www.ncbi.nlm.nih.gov/dbEST/), which serves as the reservoir of differentially expressed genes. Additionally, the relative abundance of cDNA libraries created from various plant species and organs under various physiological situations offers early insights into the expression patterns for the more abundant transcripts [15]. Several functional genomics studies have been performed to identify the abiotic stress-responsive transcripts using EST sequencing, Serial analysis of gene expression (SAGE), Super serial analysis of gene expression (SuperSAGE), and massively parallel signature sequencing (MPPS). Plant cDNA libraries were screened for identifying genes involved in abiotic stress response. Screening of the cDNA library from a salt-tolerant rice genotype showed differential expression of two genes in response to multiple stresses [52]. In *Cicer*

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arietinum (Chickpea), 5494 high-quality drought-responsive EST sequence was generated by suppression subtraction hybridization (SSH) to identify differentially expressed genes in drought-tolerant and -susceptible genotypes [53].

The SAGE is a highly competent technology that isolates unique sequence tags from individual mRNAs for transcriptome research. Although SAGE is not widely utilized in plants, it is more sensitive than EST in detecting rarely expressed transcripts [54]. SAGE helps to identify a set of specific genes to the cellular conditions and study the gene expression profile of a particular type of cells or organs. Transcriptome analysis using SuperSAGE and high-throughput sequencing has generated 17,493 SAGE UniTags from the roots of the drought-tolerant *C. arietinum* variety [55]. Another tag-based method, massively parallel signature sequencing (MPSS), has recently been introduced. It is an open-ended platform for conducting in-depth expression profiling [56]. MPSS allows the identification of millions of signatures per experiment, surpassing even the most extensive SAGE applications covers hundreds of thousands of tags. Due to more extended tags and high-throughput analysis, MPSS identifies genes with greater specificity and sensitivity [57]. Plant MPSS databases contain publicly available MPSS expression data for many plant species, including *A. thaliana* , rice, grape, *Z. mays*, and soybean [58]. MPSS databases quantify the absolute expression level of most genes. It also provides information about novel transcripts, including regulatory intergenic transcripts, alternative splice isoforms, and antisense transcripts [59].

3.2. Hybridization-Based Approaches

DNA microarrays are a advanced tool in functional genomics studies of important crop species, including *A. thaliana*, *T. aestivum*, and *O. sativa* [52,60,61]. Analysis of the microarray expression profiles is a positive approach to improve an in-depth understanding of genes involved in regulatory networks with resistance against multiple abiotic stresses. cDNA-microarrays are potent tools for improving abiotic stress tolerance in plants through various cutting-edge sequencing and bioinformatics techniques, such as miRNA-regulated networks, miRNA target prediction, miRNA identification, expression profile, Next-Generation Sequencing (NGS), and other plants specific tools [62]. In *T. aestivum*, oligo-DNA microarrays were designed to include approximately 32,000 distinctive genes characterized by several expressed sequence tags (ESTs) [61]. Stress-inducible genes cause low temperature and dehydration; their sequences were utilized to build cDNA-microarrays with 3628 descriptive exposures of the *Thellungiella salsuginea* genome developed with stress-associated gene expression [63]. In addition, microarray revealed 1886 stress-related genes as differentially regulated in RGA1 mutants in *O. sativa* [64]. Several miRNAs related to stress responses have been identified as being activated under high salinity, low temperature, and drought conditions in recent years [62].

The spotted cDNA-microarray was the most widely used technology, consisting of several PCR-amplified probes of cDNA fragments dropped, cross-linked, and dried in a matrix pattern of spots on a treated glass surface. The limitation of this approach is that designing the probe requires the information of the transcript either in the form of a sequence or a clone. In addition to that, cross-hybridization and background noise also limit their practical usefulness. Array-based data is available extensively for model plant species, but data for economically significant crop plants are limited.

3.3. Genome-Wide Association Studies (GWAS)

Genome-wide association studies (GWAS) have emerged as a powerful tool in identifying DNA variations related to the trait of interest, such as stress tolerance. GWAS provides a robust and potent tool successfully applied in germplasm collections that identifies the regulatory loci associated with resistant phenotypic traits [51]. In plant systems, there are two major approaches to studying genotype/trait associations using mapping populations, including linkage or QTL mapping and association or linkage disequilibrium (LD) mapping [65]. Association mapping is further classified into candidate gene association mapping and genome-wide association study (GWAS) [66]. Moreover, GWAS can be effectively used for fine genome-wide mapping and also provides higher

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allelic diversity at the corresponding loci and exploits historical recombination events in a population, leading to a better association between the marker and the phenotype of a desired trait. GWAS has identified 155 significant SNPs and 275 genes associated with salt sensitivity in *O. sativa* [67]. Combining QTL mapping and GWAS with transcriptome profiling complements the identification of differentially expressed candidate genes in various crop species. GWAS combination with Meta-QTL analysis can be used to investigate the critical genomic regions and major quantitative traits in *T aestivum* [68,69]. In *T. aestivum*, meta-analysis was performed using previously identified QTLs associated with abiotic stress, including drought, heat, salinity, temperature, and aluminum stress, resulting in 76 meta-QTLs verified using genome-wide association studies [70]. In *Z. mays*, 86 candidate genes and 5 SNPs related to salt tolerance were identified by GWAS [71]. Phosphorus (P) is the essential macronutrient in crop growth and production, and its deficiency is one of the major limiting factors in *G. max* production, especially at the reproductive stage. GWAS and a combination of high-density SoySNP analysis identified 27 P-efficiency-related single nucleotide polymorphisms (SNPs), which can be utilized in breeding high-P-efficiency varieties of *G. max* [72]. GWAS has been used in detecting genetic variations underlying diverse, complex traits in barley, such as cadmium stress [73], cold tolerance [74], drought tolerance [75,76], aluminum tolerance [77,78], and salinity tolerance [79,80]. Heat stress caused a significant decrease in grain nutrient content in *C. arietinum*. GWAS revealed that grain yield negatively correlated with Fe and Zn content and non-significantly with protein content. In *C. arietinum*, 181 marker-trait associated with grain nutrient content (Fe, Zn, and protein) under drought and stress conditions was identified using GWAS [81]. Similarly, GWAS revealed SNPs associated with QTLs involved in drought stress by evaluating the drought tolerance ability of horsegram (*Macrotyloma uniflorum*) germplasms [82].

4. Mechanisms of CRISPR/CAS9 Genome Editing

The CRISPR/Cas system relies on an adaptive immune system found in the genomes of bacteria and archaea to protect against the invasion of foreign plasmids or viral DNA [30]. CRISPR/Cas9 is a two-component system comprising CRISPR-associated protein 9 (Cas9) and a single guide RNA (sgRNA). CRISPR/Cas system can be divided into two classes based on the structure and functions of Cas-proteins: Class I (type I, III, and IV) and Class II (type II, V, and VI) [83]. Class 1 systems rely on multi-subunit protein complexes, whereas Class 2 systems utilize single effector proteins. Since the structure of type II CRISPR/Cas9 is relatively simple, it has been well-studied and extensively used in genetic engineering. The first Cas protein (Cas9) used in genome editing was extracted from *Streptococcus pyogenes* (SpCas9). Cas9 is a large (1368 amino acids) multi-domain DNA endonuclease that cleaves the target DNA to form a double-stranded break called a genetic scissor [84]. The mechanism of CRISPR/Cas9 genome editing can be generally divided into three steps: recognition, cleavage, and repair. The designed sgRNA directs Cas9 and identifies the target sequence in the gene of interest through its 5'crRNA complementary base pair component. The 20 nucleotides at the 5′ end of a sgRNA, as a component of the sgRNA/Cas9 complex, bind to the target genome site. The Cas9 protein remains inactive in the absence of sgRNA. The Cas9 nuclease makes double-stranded breaks (DSBs) at a site three base pair upstream to the protospacer adjacent motif (PAM). This specific target site must be located immediately upstream of the PAM, a conserved DNA sequence downstream of the cleavage site. The most commonly used nuclease in the genome-editing tool, Cas9 protein recognizes the PAM sequence at 5'-NGG-3'. Several studies have been conducte to improve the efficiency of the CRISPR/Cas system. For example, plants' unique PAM sites (NGG and NG) were discovered using SpCas9-NG and Cas9-NG variants [85]. SpCas9 orthologues have been recognized from *Streptococcus thermophiles* (St1Cas9) [86] and *Staphylococcus aureus* (SaCas9) [87]. Engineered SaCas9 has been developed to target plant genomic loci with the PAM sequence of NNNRRT [88]. Additionally, engineered SpCas9 has been developed to amend plant genomic loci with PAM sequences of NGG, NG, NRNH, NGN, NRN, or NYN [89–95] [Table 1].

Two mechanisms repair the double-strand break (DSBs) created by Cas9 protein: homology-directed repair (HDR) and non-homologous end joining (NHEJ). Homology-directed repair (HDR) is exact and requires a homologous DNA template. HDR requires several donor DNA templates with

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a target DNA sequence and is primarily active in the late S and G2 phases of cell cycle. HDR executes the specific gene insertion or gene replacement by adding a donor DNA template with sequence homology at the predicted DSB site [21]. Non-homologous end-joining (NHEJ) is the leading and efficient cellular repair mechanism and is active in all cell cycle phases. Unlike HDR, NHEJ is an error-prone mechanism that may result in indels (short insertions and deletions) at the cleavage site, leading to frameshift mutation or premature stop codon in the sequence. NHEJ accelerates the repairs created by double-strand breaks (DSBs) by joining DNA fragments using an enzymatic procedure without exogenous homologous DNA [19,20,22].

Table 1. Type of protospacer adjacent motifs (PAMs) sequences used in CRISPR/Cas genome editing system.

Name	Cas	Resources	PAM sequence	PAM location	Reference
SpCas9	Cas9	*Streptococcus pyogenes*	NGG	3′	[84]
St1Cas9	Cas9	*Streptococcus thermophilus*	NNAGAAW or NGGNG	3′	[86]
SaCas9	Cas9	*Streptococcus aureus*	NNGRRT	3′	[87]
NmCas9	Cas9	*Neisseria meningitidis*	NNNNGATT	3′	[96]
FnCas9	Cas9	*Francisella Novicida*	NGG	3′	[97]
CjCas9	Cas9	*Campylobacter jejuni*	NNNNRYAC	3′	[98]
AsCas12a	Cas12a(cpf1)	*Acidaminococcus sp.*	TTTV	5′	[25]
LbCas12a	Cas12a(cpf1)	*Lachnospiraceae bacterium*	TTTV	5′	[25]
FnCas12a	Cas12a(cpf1)	*Francisella Novicida*	TTTN or YTN	5′	[25]
LsCas13	Cas13(C2c2)	*Leptotrichia shahii*			[99]
Cas14	Cas14	Archaea			[100]
FnCas9 variant	Cas9	Modified FnCas9	YG	3′	[97]
Modified SpCas9	Cas9	Engineered SpCas9	NGA or NAG	3′	[101]
SaCas9-KKH	Cas9	Engineered SaCas9	NNNRRT	3′	[88]
SpCas9-HF	Cas9	Engineered SpCas9	NGG	3′	[89]
eSpCas9	Cas9	Engineered SpCas9	NGG	3′	[90]
SpCas9-NG	Cas9	Engineered SpCas9	NG	3′	[85]
Sniper-Cas9	Cas9	Engineered SpCas9	NGG	3′	[91]
evoCas9	Cas9	Mutated SpCas9	NGG	3′	[92]
HypaCas9	Cas9	Mutated SpCas9-HF	NGG	3′	[93]
Cas9-NRNH	Cas9	Engineered SpCas9	NRNH	3′	[94]
SpG	Cas9	Engineered SpCas9	NGN	3′	[95]
SpRY	Cas9	Engineered SpCas9	NRN or NYN	3′	[95]

"N" is any nucleotide ["A", "T", "G', "C"]. "R" is "A" or "G". "H" is "A", "C" or "T". "Y" is "C" or "T". "W" is "A" or "T". Cas13 targets RNA sequences instead of DNA; Cas14 targets single-stranded DNA (ssDNAs) instead of double-stranded DNA (dsDNAs) and does not require a Protospacer-Adjacent Motif (PAM).

The Cas9-gRNA system has been shown to function in different organisms. It has been used to generate targeted gene modifications in multiple plant species: *N. benthamiana* [29], *N. tabacum* [30], *A. thaliana* [31], *Z. mays* [32], *O. sativa* [33], *L. esculentum* [37], *S. tuberosum* [38], *T. aestivum* [34], *G. max* [41], *C. annuum* [39], *H. vulgare* [35], *S. italica* [36], and *Saccharum spp.* [42].

Two main criteria for CRISPR/Cas genome editing are efficacy and specificity. Numerous computational in silico tools have been developed for designing guide RNAs to predict cleavage efficiency and accurate target specificity (Table 2). The freely available online tools for sgRNA design and quality check are CHOPCHOP (https://chopchop.cbu.uib.no/, accessed on 23 August 2023) [102], Cas-OFFinder (http://www.rgenome.net/cas-offinder/, accessed on 23 August 2023) [103], CCTop (https://cctop.cos.uni-heidelberg.de/, accessed on 23 August 2023) [104], CRISTA (https://crista.tau.ac.il/, accessed on 23 August 2023) [105], CRISPR-GE (http://skl.scau.edu.cn/, accessed on 23 August 2023) [106], CRISPR-P (http://crispr.hzau.edu.cn/CRISPR2/, accessed on 23

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August 2023) [107], CRISPR-PLANT V2 (http://omap.org/crispr2/, accessed on 23 August 2023) [108], CRISPRlnc (http://www.crisprlnc.org/, accessed on 23 August 2023) [109], SNP-CRISPR (https://www.flyrnai.org/tools/snp_crispr/web/, accessed on 23 August 2023) [110], and PnB Designer (https://fgcz-shiny.uzh.ch/PnBDesigner/, accessed on 23 August 2023) [111].

Table 2. Computational tools for designing guide RNA 9 (sgRNA).

Tool	Organism	Major feature	Weblink
CHOPCHOP	> 100 species, including plants	Providing several predictive models and primers. Visualizing the genomic location of genes and targets [102].	https://chopchop.cbu.uib.no/
Cas-OFFinder	>100 species, including plants	Searching potential off-target sites [103].	http://www.rgenome.net/cas-offinder/
CCTop	> 100 species	Predicting off-target impacts and sgRNA efficiency using CRISPRater with custom *in vitro* transcription. Searching for single and multiple queries [104].	https://cctop.cos.uni-heidelberg.de/
CRISTA	> 100 species	Detecting off-target, providing machine learning framework, including DNA/RNA genomic context and RNA thermodynamics [105].	https://crista.tau.ac.il/
CRISPR-GE	> 40 plant species	PCR sequencing result analysis. Providing software toolkits, primer design for vector construction, and on-target amplification [106].	http://skl.scau.edu.cn/
CRISPR-P	49 plant species	Providing on-target and off-target scoring and gRNA sequence analysis [107]	http://crispr.hzau.edu.cn/CRISPR2/
CRISPR-PLANT V2	7 plant species	Allows selection of particular chromosomes and a resource for specific gRNA spacer sequences [108].	http://omap.org/crispr2/
CRISPRlnc	10 species	Provides hundreds of lncRNAs and thousands of validated sgRNA [109].	http://www.crisprlnc.org/
SNP-CRISPR	9 plants and animal species	Designing sgRNAs (NGG and NAG) for targeting SNPs or Indels [110].	https://www.flyrnai.org/tools/snp_crispr/web/
PnB Designer	*O. sativa*, *V. vinifera*	Designing sgRNAs for base editors and pegRNAs for prime editors [111].	https://fgcz-shiny.uzh.ch/PnBDesigner/

5. Impact of CRISPR/Cas9-Based Genome Editing on Abiotic Stress Tolerance

Abiotic stresses hinder plant growth and development, which can cause a 50% reduction in crop yield [112]. Though traditional breeding increases production to a large extent, it has the drawback of losing genetic variety and fitness. In addition to being time-consuming, its reliance on natural allelic variants makes it challenging to create the desired characteristic and ensure the sustainability

of production. Genome editing involves precise modifications in the genome at specific sites to make desired changes to the DNA sequence [20,21,30]. Genome editing techniques using sequence-specific nucleases (SSNs) have become popular in plant research to develop improved cultivars in terms of yield, nutrition content, and resistance to environmental stresses. The SSNs introduce DNA double-strand breaks (DSBs) at a target site, stimulating the cellular DNA repair and resulting in genome alterations, including targeted mutagenesis, gene insertion, and gene replacement [113]. In recent years, three types of genome-editing tools have been widely used, including zinc finger nucleases (ZFNs), transcription activator-like effector nucleases (TALENs), and clustered regularly interspaced palindromic repeat CRISPR/Cas protein systems. Due to complex procedures and high failure rates, ZFN and TALEN have not been utilized extensively, whereas CRISPR/Cas was successfully used in various crop improvement programs. CRISPR/Cas9-based genome editing is more precise to genomic targets and less likely to change the genomic background of a variety. With the improved bioinformatics tools and functional studies, the negative regulators of abiotic stress can also be identified and further modified using CRISPR/Cas9 tools. It also targets specific genes involved in stress response pathways and modifies them to enhance the plant's ability to withstand adverse environmental conditions [30,114]. CRISPR/Cas system has been used to introduce critical agricultural traits, including plant resistance and other agronomically important traits (increased grain size and grain weight) into many economically important crops, such as *O. sativa*, *T. aestivum*, *Z. mays*, *L. esculentum*, *S. tuberosum*, *N. tabacum*, *Gossypium spp.*, *G. max*, *Brassica sp.*, *S. italica*, and *Saccharum spp.* [5,30–34,37,39,40].

5.1. CRISPR for Drought Stress Tolerance in Plants

Drought stress can reduce crop yields by 50–70% in different crops due to significant reductions in plant growth and development. For example, 27–40% yield reduction has been observed in *C. arietinum*, 42% in *G. max*, 50% in *O. sativa*, 21% in *T. aestivum*, 68% in *V. unguiculata,* and 40% in *Z. mays* [5]. Plants experience morphological, physiological, biochemical, and molecular changes in response to drought stress. Several studies have demonstrated that increasing rice ability to withstand drought can be attained by reducing the expression of the regulatory genes *DERF1*, *PMS3*, *MSH1*, *MYB5*, and *SPP* using the CRISPR/Cas technique [115].

Overexpression of several drought-responsive genes and transcription factors increases the numbers of signaling molecules and metabolic compounds, resulting in drought tolerance in plants. Drought and other stress-related genes have been altered using CRISPR/Cas to produce drought-resistant crops. A truncated version of gRNAs (> 20 nucleotides) with target sequences in plant cells was used to improve the specificity of CRISPR/Cas9 and eventually generate altered alleles for *OST2* (Open Stomata 2). Gene *OST2* encodes *AHA1*, a major plasma membrane H+-ATPase that exhibited altered stomatal closing in response to environmental stress in *A. thaliana* [116]. Similarly, the remodeled CRISPR/Cas9 activation system activates vacuolar H+-pyro phosphatase *AVP1*, leading to increased single-leaf area, increased leaf numbers, and enhanced tolerance to drought stress [3]. Improved drought resistance was found in homozygous CRISPR/Cas9-edited *MIR169a* T3 plants using a combinatory dual-sgRNA/Cas9 vector containing deleted miRNA gene regions (*MIR169a* and *MIR827a*) [117]. Histone acetyltransferase (*HAT*) modifies chromatin histone, exposing DNA to the transcriptional machinery and regulating gene expression. Stable transgenic plants expressing chimeric *dCas9HAT* in *A. thaliana* showed higher chlorophyll content, faster stomatal aperture, and an improved survival rate under drought-stress conditions [118]. The *trehalase* (*TRE1*) gene silencing through CRISPR/Cas9 system developed drought tolerance in *A. thaliana* [119]. Transcriptome analysis using microarray technology is a best technique that has proven helpful in discovering many stress-inducible genes. These stress-inducible transcription factors include members of the DRE-binding protein (*DREB*), ethylene-responsible element binding factor (*ERF*), zinc-finger, *WRKY*, *MYB*., basic helix-loop-helix (*bHLH*), basic-domain leucine zipper (*bZIP*), *NAC* (*NAM*, *ATAF1*, and *CUC2*), and homeodomain transcription factor families [4]. Overexpressing *AtNAC07*, *AtNAC019*, and *AtNAC055* can enhance tolerance to drought in *A. thaliana* [120]. Dehydration-responsive element binding (*DREB*) proteins are one of the most prominent transcription factors and have a significant

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role in signaling networks regulating various plant development processes and stress responses. The overexpression of *DREB1A/CBF3* (C-repeat binding factor) under the stress-inducible RD29A promoter improved drought tolerance in transgenic *T. aestivum* [121]. Dehydration-responsive element binding 2 (*TaDREB2*) and Ethylene Responsive Factor 3 (*TaERF3*) were altered using the CRISPR/Cas system in *T. aestivum* [122].

Abscisic acid (ABA) plays a vital role in drought tolerance by regulating the expression of many drought-related genes. ABA regulates the expression of genes through ABA-responsive element (*ABRE*) binding protein/ABRE binding factor (*AREB/ABF*). Over-expression of *AREB1* has shown improved tolerance to drought stress as compared to *AREB1* knockout mutant [123]. In *A. thaliana, ABF1, ABF3, AREB1/ABF2, and AREB2/ABF4* are expressed in response to ABA and drought stress in vegetative tissues, whereas *ABI5, AREB3, DPBF2,* and *EEL* are expressed during seed maturation [123–125]. Abscisic acid (ABA)-induced transcription repressors (*AITRs*) regulate ABA signaling. CRISPR/Cas9 gene editing system was used to target the six *GmAITRs* simultaneously and generated Cas9-free *gmaitr36* double and *gmaitr23456* quintuple mutants, enhancing salinity tolerance in *G. max* [41]. Similarly, the Dehydration-responsive element [*DREB1]/CBF* is responsible for the ABA-independent induction of several genes in response to osmotic and cold stress. For example, *RD29A/COR78/LTI78* gene in *A. thaliana.* The lateral organ boundaries domain (*LBD*) genes play essential roles in lateral organ development. CRISPR/Cas9 knockout of *SlLBD40* improved drought tolerance in *L. esculentum* compared with overexpressing transgenic and wild-type plants [126]. Mitogen-activated protein kinases (*MAPKs*) are important signaling molecules that respond to drought stress. Similarly, the knockout mutant for the *SlMAPK3* gene down-regulated the expressions of drought stress-responsive genes: *SlLOX*, *SlGST*, and *SlDREB* [127,128]. The CRISPR-Cas9 mediated $dst^{\Delta 184–305}$ mutation in the *DST* (drought and salt tolerance) gene of *O. indica* cv. *MTU1010* produced mutants having broader leaves and reduced stomatal density, resulting in improved leaf water retention under drought stress [129]. The SNF1-related protein kinase 2 (*SnRK2*) is the primary regulator of hyper-osmotic stress signaling and abscisic acid (ABA)-dependent plant development. A knockout mutant of the *SAPK2* gene improved drought tolerance in *O. sativa* by affecting ABA signaling [130]. The CRISPR/Cas9-mediated knockout of *SRL1* and *SRL2* (Semi-rolled leaf 1, 2) and *ERA1* (Enhanced Response to ABA1) genes improved drought tolerance in *O. sativa*. *OSERA1* mutant lines display similar leaf growth as wild-type plants but enhanced primary root growth [131]. The *SRL1* and *SRL12* knockout mutants had fewer stomata, a slower rate of transpiration, less chlorophyll, vascular bundles, and rolled leaves than the wild type [132]. Plant *ITPKs* (Inositol trisphosphate 5/6 kinases) participate in abiotic stress signaling, and the *itpk1* mutant created using programmable nuclease Cas9 displayed higher tolerance to salinity stress than deletion mutants in *H. vulgare* [133]. In *B. napus*, *bnaa6.rga* mutant generated through CRISPR/Cas9 showed enhanced drought tolerance by promoting stomatal closure through increased ABA sensitivity [40].

ARGOS is a negative regulator of the ethylene response, and CRISPR/Cas9-mediated editing of the ethylene response factor *ARGOS8* improved drought tolerance in *Z. mays* [134]. *WRKY* are plant-specific transcription factors that play essential roles in abiotic stress response. Several *WRKY* transcription factors were identified in plant species, including *A. thaliana*, *O. sativa*, *G. max*, *T. aestivum*, and *H. vulgare* [135–137]. Overexpression of *ZmWRKY40* promoted root growth and reduced the water loss rates in transgenic *A. thaliana* under drought stress [136]. Overexpression of the *T. aestivum TaWRKY33* enhanced the drought and heat tolerance in transgenic *A. thaliana* [135]. *OsWRKY5* is expressed in developing leaves at the seedling and heading stages of *O. sativa*. It is the negative regulator of drought, and its expression was reduced under drought stress and by treatment with NaCl, mannitol, and abscisic acid (ABA) [137].

The potential of CRISPR/Cas gene editing has been documented in various crop species against drought stress (Table 3). Researchers aimed to enhance plants' ability to withstand drought stress and reduce crop losses by altering drought-related genes.

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Table 3. Application of the CRISPR-based genome editing approach in tailoring abiotic stress tolerant plants.

Crops	Targeted Gene	Trait	References
Arabidopsis thaliana	*OST2*	Drought tolerance	[116]
Arabidopsis thaliana	*AVP1*	Drought tolerance	[3]
Arabidopsis thaliana	*MIR169a and MIR827a*	Drought tolerance	[117]
Arabidopsis thaliana	*HAT*	Drought tolerance	[118]
Arabidopsis thaliana	*TRE1*	Drought tolerance	[119]
Arabidopsis thaliana	*NAC07, NAC019, NAC055*	Drought tolerance	[120]
Arabidopsis thaliana	*Oxp1*	Metal Stress tolerance	[13]
Arabidopsis thaliana	*DREB1A*	Drought and cold tolerance	[182]
Brassica napus	*BnaA6.RGA*	Drought tolerance	[40]
Cicer arietinum	*At4CL, AtRVE7*	Drought tolerance	[217]
Cucumis sativus	*WRKY46*	Cold tolerance	[183]
Fragaria vesca	*FvICE1*	Drought and cold tolerance	[184]
Glycine max	*AITR*	Salinity tolerance	[41]
Glycine max	*ALS1*	Resistance to chlorsulfuron herbicide	[206]
Hordeum vulgare	*ITPK1*	Salinity tolerance	[133]
Lactuca sativa	*NCED4*	Heat tolerance	[176]
Lycopersicon esculentum	*SlLBD40*	Drought tolerance	[126]
Lycopersicon esculentum	*SlMAPK3*	Drought tolerance	[127,128]
Lycopersicon esculentum	*SlHyPRP1*	Salinity tolerance	[37]
Lycopersicon esculentum	*SlCBF1*	Cold tolerance	[185]
Lycopersicon esculentum	*ZAT12*	Heat tolerance	[172]
Lycopersicon esculentum	*SlAGL6*	Heat tolerance	[173]
Lycopersicon esculentum	*CPK28, APX2*	Heat tolerance	[174]
Lycopersicon esculentum	*BZR1*	Heat tolerance	[175]
Lycopersicon esculentum	*ALS*	Resistance to chlorsulfuron herbicide	[209]
Oryza sativa	*SRL1, SRL2*	Drought tolerance	[132]
Oryza sativa	*OsDST*	Drought and salinity tolerance	[129]
Oryza sativa	*OsERA1*	Drought tolerance	[131]
Oryza sativa	*SAPK2*	Drought and salinity tolerance	[130]
Oryza sativa	*RR22*	Salinity tolerance	[158]
Oryza sativa	*miR535*	Drought and salinity tolerance	[165]
Oryza sativa	*RAV2*	Salinity tolerance	[162]
Oryza sativa	*RR9, RR10*	Salinity tolerance	[170]
Oryza sativa	*NAC67*	Drought and salinity tolerance	[171]
Oryza sativa	*NAC006*	Drought and heat tolerance	[179]
Oryza sativa	*OTS1*	Salinity tolerance	[167]
Oryza sativa	*MYB30*	Cold tolerance	[189]
Oryza sativa	*Ann3*	Cold tolerance	[187]
Oryza sativa	*PRP1*	Cold tolerance	[191]
Oryza sativa	*WSL5*	Cold tolerance	[192,193]
Oryza sativa	*HSA1*	Heat tolerance	[176]
Oryza sativa	*HAK1*	Low cesium accumulation	[198]
Oryza sativa	*LCT1, Nramp5*	Reduced cadmium accumulation	[197]
Oryza sativa	*NRAMP1*	Reduced levels of heavy metals (Cd and Pb)	[14]
Oryza sativa	*PRX2*	Potassium deficiency tolerance	[199]
Oryza sativa	*ARM1*	Increase tolerance to high Arsenic	[200]
Oryza sativa	*ALS*	Resistance to Imazethapyr and imazapic herbicides	[208]

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Oryza sativa	*ALS*	Herbicide resistance	[203]
Oryza sativa	*ALS1*	Resistance to bispyribac-sodium herbicide	[207]
Oryza sativa	*ALS*	Resistance to Sulfonylurea, imidazolinone, triazolopyrimidine, pyr-imidinyl-thiobenzoates and sulfonyl-aminocarbonyl-triazolinone herbicides	[205]
Oryza sativa	*EPSPS*	Resistance to glyphosate resistance	[211]
Oryza sativa	*C287T*	Resistance to imazamox herbicide	[210]
Oryza sativa	*ALS, EPSPS*	Herbicide resistance	[214]
Oryza sativa	*BEL*	Resistance to bentazon herbicide	[215]
Oryza sativa	*OsTubA2*	Resistance to dinitroaniline herbicide	[216]
Oryza sativa	*OsDERF1*	Drought tolerance	[115]
Triticum aestivum	*DREB1A/CBF3*	Drought tolerance	[121]
Triticum aestivum	*DREB2, ERF3*	Drought tolerance	[122]
Triticum aestivum	*HAG1*	Salinity tolerance	[142]
Zea mays	*ARGOS8*	Drought tolerance	[134]
Zea mays	*HKTI*	Salinity tolerance	[149]
Zea mays	*TMS5*	Heat tolerance	[177]
Zea mays	*ALS2*	Resistance to chlorsulfuron herbicide	[202]

5.2. CRISPR for Salinity Stress Tolerance in Plants

Genome editing and genetic engineering tools have been utilized to target genes involved in ion transport for regulating osmotic adjustment under salt stress. Soil salinity is a critical abiotic stress affecting crop productivity worldwide. Plant salt tolerance is the ultimate manifestation of several physiologic processes, including Na^+ uptake and exclusion, ionic balance (especially Na^+/K^+ ratio), and distribution [6]. In *A. thaliana*, the *SOS* signal transduction pathway (including *SOS1*, *SOS2*, and *SOS3* genes) is essential for ion homeostasis and salt tolerance. *SOS1* Na^+/H^+ antiporters and Na^+ *HKT* or Na^+/K^+ *HKT* transporters are expressed in epidermal root and xylem parenchyma cells. In epidermal root cells, it contributes to sodium efflux. In xylem parenchyma cells, it may load or unload Na^+ into the xylem depending on the salinity of the environment. *A. thaliana* mutants with altered *SOS1* exhibited significant growth inhibition under salt treatment, which was rectified in the *sos1* mutant by overexpressing *SOS1* gene under the 35S promoter. Likewise, overexpression of the *SOS1* gene in wild-type plants under 35S promoter at 100–200 mM NaCl reduced sodium accumulation in shoots, and sodium concentration in xylem sap leads to enhanced salinity tolerance in *A. thaliana* [138]. The *SOS1* gene isolated from durum wheat (*T. durum*) conferred salinity tolerance to the *sos1* mutant of *A. thaliana* [139]. Similarly, the knockout of the *AITR* family genes in *A. thaliana* enhanced tolerance to drought and salinity stress without fitness costs [7]. A gene cluster containing (*T5G46490*, *AT5G46500*, A*T5G46520*) and (NLRs; *AT5G46510*) is involved in osmotic stress tolerance, and the *A. thaliana* plants containing complete deletions or pseudogenization-induced polymorphisms in *ACQOS* and *AT5G46510* show considerable tolerance to salt stress which suggested the role of *ACQOS* in salt stress tolerance [140].

Nitric oxide (NO) plays a vital role in cyto-protection by regulating the level of ROS and inducing transcriptional changes leading to the modulation of protein function [141]. Reactive oxygen species (ROS) are highly reactive molecules that are typically produced in response to environmental stress, such as salinity and drought. Histone acetyltransferase *TaHAG1* is a vital regulator to strengthen the salt tolerance of *T. aestivum*. *TaHAG1* contributed to salt tolerance by modulating ROS production and signal specificity. Moreover, *TaHAG1* directly targeted a subset of genes responsible for hydrogen peroxide production, and enrichment of *TaHAG1* triggered increased H3 acetylation and transcriptional upregulation of these loci under salt stress [142]. Salt stress increases ROS production, which is responsible for oxidative damage, membrane injury, lipid peroxidation (malondialdehyde), and ultimately cell death. CRISPR/Cas9-mediated mutagenesis of

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the *osbhlh024* gene negatively regulates the functions of Na^+ and K^+ transporter genes, suppressing the higher accumulation of MDA and H_2O_2, leading to salt tolerance in *O. sativa* [143].

Several quantitative trait loci (QTLs) and genes associated with regulating salt stress tolerance have been identified in *O. sativa*, including the *NHX* family (*OsNHX1*, *OsNHX2*, *OsNHX3*) [144–146], *HKT* family (*OsHKT1*, *OsHKT2*, *OsHKT7*) [147–150], *DCA1* [151], *DST1* [129,152], *OsKAT1* [153], *OsBADH1* [154], *OsNAC5* [155], *OsbZIP71* [156], *SKC1*, *OsHAL3*, *P5CS*, *SNAC2*, *OsNAP*, *OsRRY* [157,158], and *OsSALP1* [112,159]. CRISPR/Cas9-mediated knockout of several salt stress genes significantly improved salinity tolerance in various crops. CRISPR/Cas9 and third-generation hybrid rice system approaches were employed to generate the *OsRR22* mutant, which exhibited enhanced salinity tolerance without any morphological and physiological changes relative to the wild-type [158]. A receptor-like kinase gene *OSBBS1*/*OsRLCK109* played vital roles in leaf senescence and salt stress response [160]. CRISPR/Cas9-mediated editing of the *SAPK1* and *SAPK2* genes showed resistance to salt stress in *O. sativa* [130]. The mutant alleles of *DST* (drought and salt tolerance) generated using the *CRISPR*/Cas9 method showed reduced stomatal density by downregulating stomatal developmental genes (*SPCH1*, *MUTE*, *ICE1*), resulting in a high level of salt tolerance in the seedling stage of *O. sativa* [129]. Argonaute (*AGO*) proteins primarily function in gene silencing by forming RNA-induced silencing complexes. CRISPR/Cas9 mediated *AGO2*-knockout mutant lines showed few morphological changes compared to wild-type rice. The overexpression of *AGO2* under the control of the cauliflower mosaic virus 35S led to a simultaneous increase in salt tolerance and grain length [161]. Transcription factors such as *AP2*/*ERF*, *NAC* (*NAM*, *ATAF1/2*, *CUC2*), and *WRKY* families induce stress-responsive gene expression in response to environmental signals. APETALA2/ethylene response factor (*AP2*/*ERF*) plays crucial roles in transcriptional regulation and defense response against biotic and abiotic stress. CRISPR/Cas9-mediated editing of *OsRAV2* (AP2/ERF domain-containing RAV) gene showed tolerance to salt stress [162]. *DOF* transcription factor (DNA-binding with one finger) positively regulates primary root elongation by regulating cell proliferation in the root meristem via restricting ethylene biosynthesis. *O. sativa* mutant *osdof15* showed reduced cell proliferation and primary root elongation in the root meristem [163]. A knockout mutant (*ospqt3*) with CRISPR-Cas9 technology displayed greater resistance to oxidative and salt stress with high expression of *OsGPX1*, *OsAPX1*, and *OsSOD1* [164]. Similarly, CRISPR/Cas9 knockout of *OsmiR535* demonstrated salinity tolerance in *O. sativa* against NaCl, ABA, dehydration, and PEG stresses [165]. *OsNAC45* plays a vital role in ABA signal responses, and overexpression of *NAC45* enhances salt tolerance in *O. sativa*. *OsNAC45* may regulate the expression of seven genes namely *CYP89G1*, *DREB1F*, *EREBP2*, *ERF104*, *PM1*, *SAMDC2*, and *SIK1* [166]. Targeted mutagenesis of the *OsOTS1* gene using the CRISPR/Cas9 system in the *O. sativa* cv. Kitaake enhanced sensitivity to salt with reduced root and shoot biomass, indicating that *OsOTS1* has a major role in salt stress tolerance [167].

Hormonal signaling pathways are involved in salt stress responses. *OsPIL14-SLR1* (Phytochrome Interacting Factor-Like14 - DELLA protein, SLENDER RICE1) integrates light and GA (Gibberellic Acid) signaling to control seedling growth in response to salt stress. CRISPR/Cas9 mediated *ospil14* mutants did not produce abnormal mesocotyls but had slightly longer roots than wild-type plants [168]. In *Z. mays*, *ZmWRKY114* functions as a negative regulator of salt-stress responses, and overexpressed *WRKY114* exhibited reduced salt-stress tolerance and ABA sensitivity by regulating stress- and ABA-related gene expression [169]. Salinity stress tolerance was identified in several stress-related genes like *HyPRP1* (Hybrid proline-rich protein 1), *HKT1*, *HKT1* (High-affinity potassium transporter1;2), *RAD51/54* (DNA repair and recombination protein 51/54) and *PR-1* (Pathogenesis-related protein 1) [37,150]. Proline-rich proteins (*PRPs*) are involved in cell-wall signaling, plant development, and stress responses. *HyPRP1* is a negative regulator of salt stress responses. CRISPR-Cas9 mediated genome editing of *HyPRP1* in *L. esculentum* resulted in precise eliminations of its functional domains, proline-rich domain (*PRD*), and eight cysteine-motif (*8CM*). Plants carrying either the domains of the PRD removal variant (*PR1v1*) or 8CM removal variants (*PR2v2* and *PR2v3*) showed improved germination as compared to wild type under osmosis stress [37]. A significant improvement in Homology-directed repair (HDR) using CRISPR/LbCpf1-

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geminiviral multi-replicons was reported to target marker-free salt-tolerant *HKT1*, *HKT2* alleles in *L. esculentum* [150]. Self-pollinated offspring plants carrying the *HKT1*, *HKT2* allele showed stable inheritance and germination tolerance under salt stress conditions (100 mm NaCl concentration). In *Z. mays*, Na^+ Content1 (*ZmNC1*) encodes an HKT-type transporter *ZmHKT1*, preferentially expressed in root stele. CRISPR-Cas9 knockout lines of *ZmHKT1* increase Na^+ concentration in xylem sap and cause increased root-to-shoot Na^+ delivery, indicating that *ZmHKT1* promotes leaf Na^+ exclusion and salt tolerance by withdrawing Na^+ from the xylem sap [149] Mutations in genes *OsRR9* and *OsRR10* enhanced salinity tolerance but reduced panicle and spikelet numbers per panicle in *O. sativa* [170]. Plant-specific transcription factors, *NAM*, *ATAF1/2*, and *CUC2*, significantly control vital growth and developmental processes. Overexpression of finger millet (*Eleusine coracana*) transcription factor *OsNAC67* enhanced tolerance to high drought and salinity tolerance in *O. sativa* [171] (Table 3).

5.3. CRISPR for Heat Stress Tolerance in Plants

Heat stress is the third most crucial abiotic factor that adversely affects the yield and quality of plants during entire growth stages, from germination to harvesting. Plants respond to heat stress in various ways, including alterations in enzymes that generate reactive oxygen species (ROS), heat shock proteins (HSPs), and genes encoding scavenger proteins [8]. The advancement of structural and functional genomics technologies in plants has led to the identification and characterization of various temperature-stress-related genes to enhance plant ability to withstand heat [9]. CRISPR/Cas9 is a cutting-edge technology to understand the molecular mechanisms associated with heat stress tolerance. *ZAT12* is a member of stress-responsive C2H2 type zinc finger protein (ZFP) reported to regulate the expression of stress-activated genes mediated via ROS in plants. The transformed *L. esculentum* lines *ZT1* and *ZT5* exhibited tolerance to heat shock, high yield, improved electrolyte leakage, and chlorophyll level with a higher expression of antioxidant enzymes viz. ascorbate peroxidase, catalase, superoxide dismutase, and glutathione reductase under drought or heat-stress [172]. Similarly, CRISPR/Cas9-based genome editing of the heat-sensitive gene, Slagamous-Like 6 (*SIAGL6*), showed increased fruit setting under heat stress conditions in *L. esculentum* [173]. CRISPR/Cas9 editing of the *CPK28* (Calcium-dependent protein kinase 28) gene targeting *APX2* (Ascorbate peroxidase 2) improved heat stress tolerance in *L. esculentum* [174]. Brassinazole Resistant 1 (*BZR1*) is involved in thermo-tolerance by regulating the Feronia (FER) homologs. CRISPR/Cas9-based *bzr1* mutants showed reduced H_2O_2 production in apoplast and heat tolerance by declined Respiratory Burst Oxidase Homolog 1 (*RBOH1*). Overexpression of *BZR1* enhanced the production of apoplastic H_2O_2 and heat stress responses through RBOH1-dependent ROS signaling [175]. Photosynthetic apparatus is highly susceptible to thermal damage. Heat-sensitive albino1 (*hsa1*) mutant harbors a recessive mutation in a gene encoding fructokinase-like protein2 (*FLN2*), resulting in a severe albino phenotype with defects in early chloroplast development. CRISPR/Cas-mediated *HSA1* (heat-stress sensitive albino 1) mutants of *O. sativa* showed increased sensitivity to heat stress but had a faster greening phenotype than wild-type plants [176]. Knockout of the *ZmTMS5* gene of *Z. mays* using the CRISPR/Cas9 system generated homozygous T1 *tms5* thermosensitive male-sterile plants that are male-sterile at 32°C but are male-fertile at 24°C [177]. *NCED4* (9-cis-Epoxycarotenoid Dioxygenase4) is a key regulatory enzyme in the biosynthesis of abscisic acid (ABA). Similarly, stable homozygous *NCED4* mutants generated using CRISPR/Cas9 were capable of germinating seeds at a higher temperature (>70% germination at 37°) in Lettuce (*Lactuca sativa*) [178]. Another transcription factor, *OsNAC006*, is regulated by H_2O_2, cold, heat, abscisic acid (ABA), indole-3-acetic acid (IAA), gibberellin (GA), NaCl, and polyethylene glycol (PEG) in *O. sativa*. Furthermore, the knockout of *OsNAC006* using the CRISPR-Cas9 system resulted in drought and heat sensitivity in *O. sativa* [179] (Table 3).

5.4. CRISPR for Cold Stress Tolerance in Plants

Cold stress due to chilling and freezing temperatures hinders plant growth and development. Low temperature inhibited plant metabolic activities, producing osmotic and oxidative stress [5]. Mechanical damage and metabolic dysfunction caused by freezing temperatures reduced plant

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growth and development. In *A. thaliana*, the two subclasses, namely *DREB1/CBF* and *DREB2*, are induced by cold and dehydration, respectively [4]. Expression of *T. aestivum TaICE41* and *TaICE87* in transgenic *A. thaliana* activated the expression of *COR* genes and consequently led to the enhancement of cold tolerance, but only after cold acclimation [180]. The overexpression of *AtDREB1A* under the RD29A promoter conferred increased drought and freezing tolerance to transgenic *A. thaliana* plants without affecting growth and development [181]. Several studies have demonstrated that *WRKY* transcription factors are essential in cold, heat, drought, and salinity stress [182]. In Cucumber (*Cucumis sativus*), *CsWRKY46* is a *WRKY* transcription factor that confers cold resistance in transgenic-plant by controlling cold-stress responsive genes in an ABA-dependent manner. Overexpression of *CsWRKY46* regulates freezing and chilling resistance and increases the expression of stress-inducible genes, including *RD29A* and *COR47* [183]. In strawberry (*Fragaria vesca*), *FvICE1* is a positive regulator of cold and drought resistances, and overexpressed *FvICE1* gene improved cold and drought tolerance at the phenotypic and physiological levels [184]. *A. thaliana HOS1* (High Expression of Osmotically Responsive Genes 1) is a Ring finger E3 ubiquitin ligase, a key regulator of cold signaling. CRISPR/Cas9-mediated knockout of the *HOS1* gene showed abiotic stress tolerance, accumulation of secondary metabolites, and expression of the biosynthetic genes [10].

The C-repeat binding factor (CBF) are highly conserved CBF cold-response-system components in many plant species. It has a major role in cold acclimation and freezing tolerance in response to low temperatures. CRISPR–Cas9-mediated *SlCBF1* mutagenesis reduced chilling tolerance of *L. esculentum* because of higher electrolyte leakage, increased indole acetic acid contents, decreased abscisic acid, methyl jasmonate, and down-regulated CBF-related genes [185]. Similarly, CRISPR–Cas9-mediated mutagenesis of *CGFS*-type *GRXs* (*SlGRXS14*, *SlGRXS15*, *SlGRXS16*, and *SlGRXS17*) genes showed the sensitivity of *Slgrxs* mutants to various abiotic stresses as compared to wild-type in *L. esculentum* [186]. Plant annexins are Ca2+-dependent phospholipid-binding proteins that play a role in development and protection from environmental stresses. CRISPR/Cas9-mediated knockout mutant of annexin gene *OsAnn3* decreased cold tolerance in *O sativa* [187]. *OsMYB30* confers cold sensitivity by interacting with an *OsJAZ9* protein and downregulating the expression of β-amylase genes in *O. sativa* [188]. Novel mutants were generated by simultaneously editing three genes, *OsPIN5b* (panicle length gene), *GS3* (grain size gene), and *OsMYB30*, using CRISPR–Cas9 system showed higher yield and excellent cold tolerance [189]. PYR1]/PYR1-like [PYL]/regulatory components of the ABA receptor detects abscisic acid during abiotic stress. CRISPR/Cas9 technology was used to edit *PYL1–PYL6* and *PYL12* (group I) and *PYL7–PYL11* and *PYL13* (group II) genes of *O. sativa* [190]. A knockout mutant of the *OsPRP1* gene of *O. sativa* generated by CRISPR/Cas9 demonstrated less antioxidant enzyme activity and accumulated lower levels of proline, chlorophyll, abscisic acid (ABA), and ascorbic acid (AsA) content relative to wild-type plants under low-temperature stress [191]. CRISPR/Cas9-mediated base editing technology generated the point mutations in two genes (*OsWSL5* and *OsZEBRA3*) in protoplasts and regenerated plants of *O. sativa*. *OsWSL5* encodes a novel chloroplast-targeted pentatricopeptide repeat protein essential in rice chloroplast biogenesis under cold stress [192,193] (Table 3).

5.5. CRISPR for Metal and Herbicide Stress Tolerance in Plants

Plants suffer oxidative stress upon contact with heavy metals, leading to cellular injury. Heavy metals, including arsenic (As), copper (Cu), cobalt (Co), cadmium (Cd), iron (Fe), manganese (Mn), nickel (Ni), zinc (Zn), mercury (Hg), lead (Pb) have accumulated in soils as a result of various human activities such as the overuse of agricultural chemicals (fertilizer, herbicides, and pesticides), improper disposal of industrial and sewage waste [11]. Plants have developed detoxification mechanisms to reduce heavy metal exposure's damaging effects and accumulation. *OXP1* is an enzyme involved in 5-oxoproline metabolism and the glutathione recycling pathway. The oxp1/CRISPR tolerated plants tolerated heavy metals, such as cadmium and amisulbrom (a sulfonamide compound) [13]. Cd stress activates the antioxidant defense system and increases the production of abscisic acid (ABA), glutathione (GSH), salicylic acid (SA), jasmonic acid (JA), and

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nitric oxide (NO) [194,195]. Absorption of Cd by the roots is mediated by *O. sativa* genes (*OsNramp1*, *OsCd1*, and *OsNramp5*). In *O. sativa*, *OsHMA2*, *OsCCX2*, and *CAL1* regulate Cd transport to the xylem, and *OsHMA3* negatively regulates Cd xylem loading. Manipulation in the expression of these genes through CRISPR/Cas9 can minimize the Cd concentration in *O. sativa* [196]. Knockout mutants of *OsLCT1* and *OsNramp5* generated through CRISPR/Cas9 displayed reduced levels of Cd in *O. sativa* [197]. *OsNRAMP1* modulates metal ion and reactive oxygen species (ROS) homeostasis. *Osnramp1* mutants generated through CRISPR/Cas9 displayed reduced levels of heavy metals (Cd and Pb) in leaves and grains of *O. sativa* [14]. The *OsHAK1* gene controls the uptake and translocation of cesium [Cs+] in *O. sativa*. CRISPR/Cas9 knockout mutant of *OsHAK1* (Cs+-permeable K+ transporter) exhibited a significant reduction of Cs+ uptake in *O. sativa* [198]. Potassium [K+] is a critical macronutrient for plant growth and development. ROS was strongly induced and accumulated in K+-deficient plants. Gene *Prxs* have been involved in the toxic reduction and intracellular H_2O_2 scavenging. The overexpression of *OsPRX2* produced K+ deficiency tolerance by closing the stomata in *O. sativa* [199]. *OsARM1* (Arsenite-Responsive MYB1) is the R2R3 MYB transcription factor that regulates arsenic-associated transporters genes in *O. sativa*, and the knockout mutant (*osarm1*) generated using CRISPR/Cas system displayed improved tolerance to arsenic [200].

Herbicides destroy weeds and crop plants by interfering with or altering their metabolic processes, resulting in low yield. Thus, herbicide tolerance is one of the essential traits of crop plants that improve farming techniques and crop productivity. CRISPR/Cas-mediated genome editing techniques efficiently modify target genes and hold great potential in engineering plants with herbicide resistance [201]. In recent years, CIRSPR-Cas9-based technology has been used to generate herbicide-tolerant crops, including *O. sativa*, *Z. mays*, and *G. max* [202,203,205]. Acetolactate synthase (*ALS*) catalyzes the step in the biosynthesis of the branched-chain amino acids, including leucine (Leu), isoleucine (Ile), and valine (Val). Enzyme *ALS* is the target enzyme for two classes of herbicides: sulfonylurea and imidazolinone. Tolerance to *ALS*-inhibiting herbicides has been developed using a genome editing system in *A. thaliana*, *O. sativa*, *T. aestivum*, *Z. mays*, *S. lycopersicon*, and *Saccharum spp.* [42,202–207]. Herbicide-resistant plants were generated through CRISPR/Cas9-mediated homologous recombination of *ALS* in *O. sativa* [203]. Similarly, editing the *ALS2* gene (substitution P165 with Ser) using either single-stranded oligonucleotides or double-stranded DNA vectors as repair templates enhanced chlorsulfuron resistance in *Z. mays* [202]. Moreover, P171F substitution in the *OsALS1* allele was introduced into the *O. sativa* cultivar Nangeng 46 through precise base editing and sgRNA, resulting in tolerance to the herbicide bispyribac-sodium [207]. Four different missense mutations (P171S, P171A, P171Y, and P171F) in the P171 codon of the *ALS* gene showed different degrees of tolerance towards five typical herbicides (Sulfonylurea, imidazolinone, triazolopyrimidine, pyr-imidinyl-thiobenzoates, and sulfonyl-aminocarbonyl-triazolinone) belongs to five chemical families of *ALS* inhibitors in *O. sativa* [205]. A novel allele (*G628W*) developed from a G-to-T transversion at position 1882 of *OsALS* gene and conferred resistance to herbicide stress. These mutant plants of rice conferred resistance to Imazethapyr (IMT) and imazapic (IMP) herbicides [208]. The CRISPR/Cas9 system was also successfully used to edit the *ALS1* gene of *G. max* to obtain chlorsulfuron-resistant plants [206]. Mutation of the Proline-186 residue in the *ALS* gene conferred chlorsulfuron resistance in *L. esculentum* [209]. Activation-induced cytidine deaminase (AID) is an enzyme that converts C to U in DNA/RNA by deamination. Target-AID is a synthetic complex of nuclease-deficient Cas9 fused to an activation-induced cytidine deaminase that enables targeted nucleotide substitution (C to T or G to A). The point mutation C287T of the *ALS* gene in rice plants resisted the herbicide imazamox [210]. In *T. aestivum*, herbicide tolerance plants were generated by base editing the acetolactate synthase (*ALS)* and acetyl-coenzyme A carboxylase genes conferred resistance to sulfonylurea, imidazolinone- and aryloxy phenoxy propionate-type herbicides [204]. Co-editing three copies of the *ALS* gene resulted in herbicide tolerance in *Saccharum spp.* [42].

Glyphosate is one of the well-known and broad-spectrum herbicides used in the weed management of resistant crops, such as *C. annum, G. max, O. sativa,* and *Z. mays*. Glyphosate inhibits the enzyme *EPSPS* (5- enolpyruvyl shikimate 3-phosphate synthase), responsible for the biosynthesis of aromatic amino acids and secondary metabolites. Site-specific gene replacements and insertions in

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the rice endogenous *EPSPS* gene resulted in glyphosate-resistant plants [211,212]. CRISPR/Cas9 tool creates a structural variation (genomic duplication or inversion) in chromosomes. The resulting mutant developed through CRISPR/Cas technology showed the high transcript accumulation of *CP12* and *Ubiquitin2* genes and the 10th fold upregulated expression of *HPPD* (4-hydroxyphenyl pyruvate dioxygenase) and *PPO1* (protoporphyrinogen oxidase) resulted in herbicide resistance without affecting the yield and other agronomically important traits in *O. sativa* [213]. CRISPR-Cas9 system was used to edit the target genes of herbicides (*ALS* and *EPSPS*) in *L. esculentum* cv. Micro-Tom [214]. Another herbicide resistance gene, Bentazon Sensitive Lethal (*BEL*), confers resistance to bentazon and sulfonylurea herbicides in *O. sativa*. CRISPR/Cas9-based mutation of the *BEL* gene was evaluated in rice using the *Agrobacterium*-mediated stable transformation [215]. The mutagenesis efficiency ranged from 2% to 16%, and the phenotypic analysis revealed that the mutated transgenic plant was sensitive to the herbicide bentazon. Precise editing of the endogenous α-tubulin homolog gene *OsTubA2* using CRIPSR-mediated adenine base editors at the T1981 site. The point mutation in the *OsTubA2* gene transformed the *O. sativa* cultivar into a herbicide (dinitroaniline) tolerant cultivar [216] (Table 3).

6. Conclusions and Future Perspectives

CRISPR/Cas genome editing tool provided a unique and innovative approach to produce resistant crops against environmental stress. It is considered the best genome editing method compared to ZFNs and TALENs due to its simplicity, low cost, and high efficiency. CRISPR/Cas can be used for gene knockout, knockdown, point mutation, replacement, and insertion, leading to development of abiotic stress-tolerant plants. Multiplex genome editing systems can edit a single gene or a few genes, but editing polyploid crops is challenging because their entire genomes have been duplicated or triplicated. The availability of plant genome sequences allows researchers to tailor the genome precisely, facilitating the use of CRISPR/Cas9 in resistance breeding. However, some limitations are associated with this CRISPR/Cas system, such as off-target, which can be reduced by carefully designing sgRNAs and using specific nucleases. PAM sequence provides target specificity and guides the genome editing sites. The application of CRISPR/Cas system will increase with the development of PAM-independent CRISPR/Cas system in crop improvement. Numerous studies demonstrate that Cas enzyme modifications may decrease the PAM requirement and create a novel tool for gene functional studies. Several efficient computational tools, including CRISPR-GE, CRISPR-P, and CRISPR-PLANT-V2, have been developed to design the guide RNA precisely. Off-target effects also be reduced by direct delivery of the protein or RNA of Cas enzymes and sgRNAs by RNP transformation.

The application of a multiplex genome editing system would open the opportunity to develop resistant crops against multiple traits in a single transformation event. Several methods, including *Agrobacterium*-mediated transformation or particle bombardment, are used for genetic manipulation, but many crops are recalcitrant or exceptionally hard to transform. In order to build an effective tissue culture and plant regeneration system, an attempt has been made to culture immature embryos that have potentials to convert into a complete plant. Development of transgene-free crops takes longer due to several repeated back-crossings of the edited plant. The commercialization and regulation of genome-edited crops are highly debated and vary across countries, which can make the commercialization process lengthy and expensive. Genome-edited products are treated equally to genetically modified organisms (GMOs) and regulated in a similar way in many countries. A uniform regulatory framework is required to facilitate the safe and efficient use of genome editing in crop improvement.

CRISPR/Cas-based genome editing holds great potential for both fundamental and applied research, which has been becoming a powerful tool for resolving important biological and agricultural problems. The fundamental areas of plant research and improvement will depend on accurate information about the various aspects of the CRISPR/Cas9 system, such as high-throughput genome sequencing method, guide RNA designing tools, and plant cell transformation and regeneration protocols.

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