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Review

Application of CRISPR-Cas9-Mediated Gene Editing for Abiotic Stress Management in Crop Plants

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Abstract: Abiotic stresses, including drought, salinity, cold, heat, and heavy metals, extensively reduce global agricultural production. Approaches such as conventional breeding and transgenic breeding have been widely used to cope with these environmental stresses. The clustered regularly interspaced short palindromic repeat- Cas (CRISPR/Cas) based gene-editing tool has revolutionized due to its simplicity, accessibility, adaptability, flexibility, and wide applicability. This system has a great potential to build up crop varieties with enhanced tolerance against abiotic stresses. In this review, we summarize the most recent findings on understanding the mechanism of abiotic stress response in plants and the application of CRISPR/Cas mediated gene-editing system towards enhanced tolerance to drought, salinity, cold, heat, and heavy metals stresses. Furthermore, in this review, we highlighted the recent advancements in prime editing and base editing tools for crop improvement.

Keywords: abiotic stress tolerance; base editing; CRISPR/Cas9; crop production; gene editing; prime editing

1. Introduction

Abiotic stresses such as drought, heat, cold, salt, and metals are the major challenges that negatively affect agro-ecological conditions and agricultural production [1]. These abiotic stressors are the key growth factors that broadly limit the productivity and quality of crop plants globally [2,3]. These abiotic factors lead to several changes at the plant's morphological, physiological, biochemical, and molecular levels, resulting in yield and production losses [4]. To feed the projected worldwide population of 9.7 billion by 2050, the estimated agricultural production will have to be enhanced by at least 85% [5,6]. Although, the conventional breeding approach has notably contributed to developing abiotic stress tolerance in crops. However, the conventional breeding approach to enhance abiotic stress tolerance is an important way to increase crop yield.

Conversely, this method may take several years to decades to increase abiotic stress tolerance [7]. Therefore, additional efficient and latest technologies with instant impacts are certainly required to deal with these challenges [8]. Genome editing (also called gene editing) tools provide a method to change an organism's DNA through introducing targeted mutation, insertion/deletion (indel), and specific sequence alteration via specific nucleases. During the past years, Meganucleases [9], transcription activator-like nucleases (TALENs) [10] zinc-finger nucleases (ZFNs) [11], and CRISPR–Cas9 [12] have been

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developed and used for genome editing. The clustered regularly interspaced short palindromic repeats (CRISPR)/CRISPR-associated protein 9 (CRISPR/Cas9) system is the most advanced and successful technology of genome editing in a wide range of organisms, including plants [13]. It is more efficient, cheaper, faster, and more accurate than other genome editing tools. In addition, this system can detect and cleave complementary DNA sequences in the genome. It was adapted from a naturally occurring gene-editing system in bacteria to provide resistance against invading viruses. However, it is presently accepted as part of an adaptive defensive system that includes CAS enzymes associated with CRISPR/Cas9 [14]. This technology may further help enable and promote using the CRISPR/Cas9-based products to overcome societal acceptance issues due to the 'foreign DNA' approach. However, few countries have adopted genome-edited crops; conversely, several other countries are still debating this subject. Recently, CRISPR/Cas9 edited tomato, which contains higher amounts of γ-aminobutyric acid (GABA) than non-edited counterparts, has been commercialized in Japan's market [15]. This technology is projected to go a long way toward enabling a comparatively painless acceptance of genome-edited crops in most countries.

CRISPR/Cas9 system has been effectively applied in several plant species including model plants such as *Nicotiana benthamiana* [16], *Nicotiana tabacum* [17,18], Arabidopsis [16,19] and in crop plants such as wheat [20], maize [21], rice [20,22] liverwort [23], tomato [24], potato [25], soybean [26], sweet orange [27], banana[28], pepper [29], and sugarcane[30]. Additionally, CRISPR-Cas9-based multiplexing by targeting multiple genes in a single organism has also been carried out successfully in various crops such as wheat [31], rice [22], cotton [32] and maize [33]. Therefore, this technology has huge potential to produce genome-edited crop plants tolerant to multiple stresses by targeting numerous stress-sensitive genes concurrently in an elite high-yielding but sensitive cultivar and tolerance genes also be overexpressed using CRISPR-mediated gene activation as well [34]. Mushtaq et al. [35] reported that the CRISPR/Cas-based gene-editing tool could efficiently target complex quantitative genes associated with abiotic stresses directly or indirectly. In rice, several genes, including betaine aldehyde dehydrogenase (*OsBADH2*), mitogen-activated protein kinase (*OsMPK2*), stress/ABA-activated protein kinase 2 (*SAPK2*), and phytoene desaturase (*OsPDS*) were found to be implicated in abiotic stress tolerance using the CRISPR/Cas9 mediated gene editing [20,36]. In plants, abiotic stress tolerance was improved through gene editing of ethylene responsible factor (ERF, a transcriptional factor) of the AP2/ERF superfamily [37].

Currently, CRISPR/Cas-based genome engineering has been proficiently used to understand tolerance against multiple abiotic stresses, including drought, salinity, heat, and nutritional values in various important crop plants [38,39]. In this review, we summarize most potential applications of the CRISPR/Cas9 mediated genome editing approach in crop plants for managing abiotic stresses such as drought, salinity, heat, etc., and discuss the future projection of this tool for the development of stress-tolerant crop varieties.

2. Mechanistic overview of CRISPR-Cas9-based genome editing technology

CRISPR/Cas system is based on an adaptive immune system discovered in bacterial and archaeal genomes to protect against the invasion of foreign plasmids or viral DNA [40]. CRISPR/Cas9 system has the CRISPR-associated protein 9 (Cas9) and a single guide RNA (sgRNA) as an essential component [41]. The sgRNA is a synthetic combination of two different RNAs necessary for CRISPR activity, the protospacer-matching CRISPR RNA (crRNA) and the transactivating crRNA. The 20 nucleotides at the 5′ end of a sgRNA as a component of the sgRNA/Cas9 complex, which binds to the target genome site. This specific target site must be located immediately upstream of the protospacer adjacent motif (PAM; NGG for SpCas9 from *Streptococcus pyogenes*), i.e., a short (typically 2-5 base-pair length) conserved DNA sequence downstream to the cleavage site and its size alters based on the bacterial species. The SpCas9 protein is a large (1368 amino acids) multi-domain DNA endonuclease accountable for the cleavage of target DNA in the genome and

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produces a blunt-ended double-strand break (DSB) and is called a genetic scissor. Finally, the DSB is repaired by the host cellular machinery [42].

Double-Stranded Breaks formed by Cas-9 protein are repaired by two pathways, i.e., homology-directed repair (HDR) and non-homologous end joining (NHEJ) mechanisms [43]. Homology-directed repair is exceptionally accurate, and it employs a homologous DNA template. HDR is mainly active in the cell cycle's late S and G2 phases and needs a large amount of donor DNA templates containing a target DNA sequence. It implements the specific gene insertion or replacement by adding a donor DNA template with sequence homology at the predicted DSB site [43,44]. Non-homologous end-joining expedites the DSBs repairs by joining DNA fragments using an enzymatic procedure without exogenous homologous DNA. NHEJ is usually active in all cell cycle phases and is a competent repair mechanism mainly active in the cells. NHEJ leads to somatic cells and typically results in altered products having short base insertions or deletions (indels), therefore inducing mutations to the targeted genes [45]. However, it is a vulnerable process that may create little random insertion or deletion (indels) at the cleavage site leading to the generation of frameshift mutation or premature stop codon [44]. The schematic representation of mechanistic insights of CRISPR-Cas9-based genome editing in plants is given in **Figure 1**.

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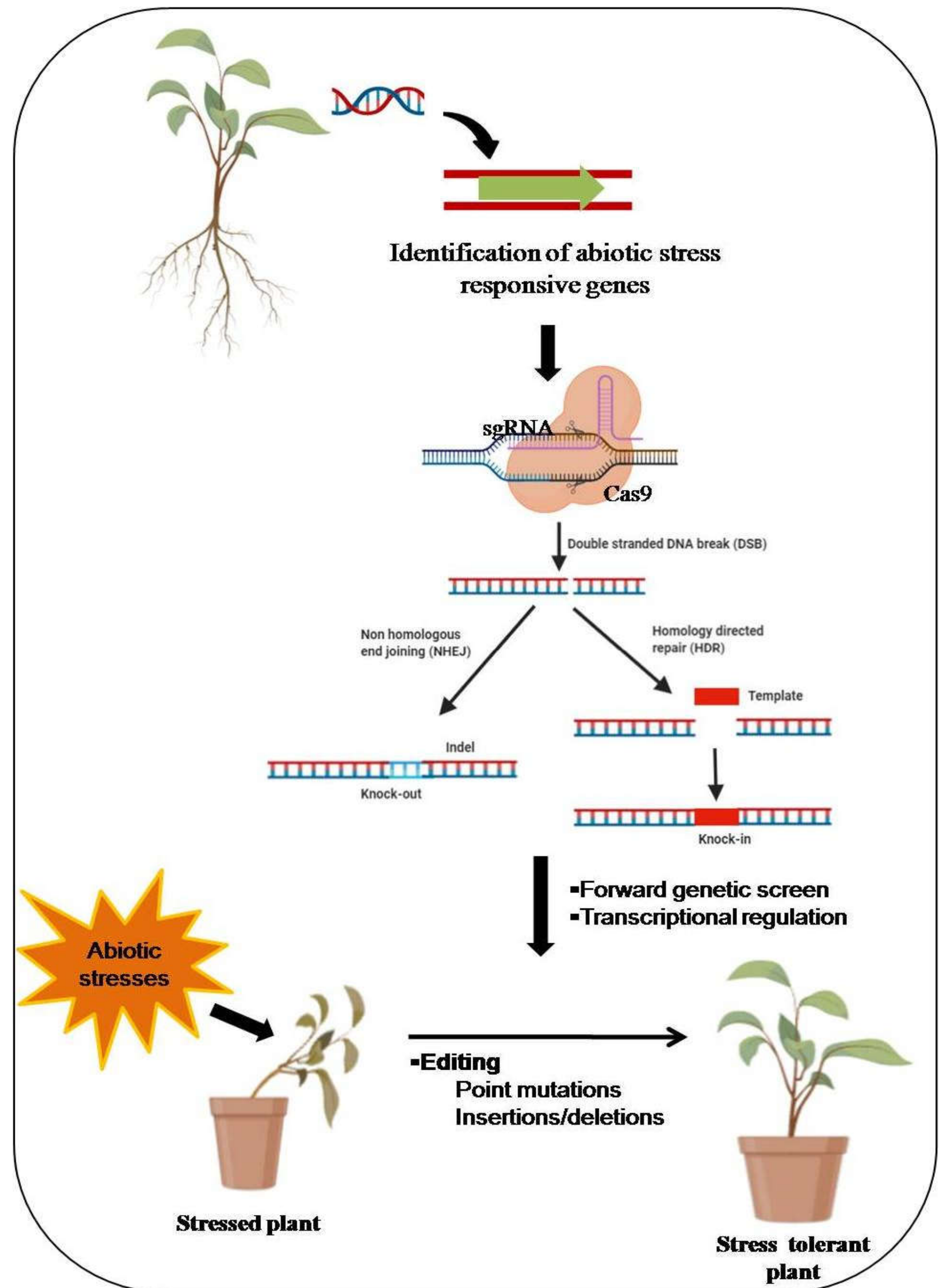

Figure 1. Schematic display of mechanistic insights of CRISPR-Cas9-based genome editing in plants. The Cas9 protein is guided by a desired single guide RNA (sgRNA) and creates a double-strand break (DSB). Subsequently, DNA repair occurs through non-homologous end-joining (NHEJ) or homology-directed repair (HDR) pathways. Figure created with BioRender.com (https://app.biorender.com/biorender-templates)-accessed on 25 May 2022.

3. Novel technological approaches and strategic suggestions for genome editing

Novel gene editing approaches evolved from the CRISPRs-Cas9, base editing (BE), and prime editing (PE) technologies open new perspectives for the functional analysis of genes. The editing efficiency of Cas9 could be improved through competent screening of targeted characteristics, investigating genetic material through gene knock-out, and using an ultimate genetic transformation procedure. Here, we present the novel breakthrough to enhance genome editing in crop plants. These strategies have been summarized,

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including base editing and prime editing, transgene-free genome editing, production of mutant libraries, and multiplexed CRISPR technologies for gene editing.

3.1. Base editing and prime editing

Many important crop traits can be improved with a single base change in the genes and do not require the events of DNA DSBs and donor DNA templates for HDR. Single base editing in such situations cannot be accomplished with the knock-in/out approach in the regular CRISPR-Cas system. Reports of agronomic traits discovered that numerous such traits are resolute by changes in the single bases of genes [46]. Regrettably, the CRISPR/Cas9 system has limitations and cannot be used to carry out gene base conversion. Therefore, it is the most suitable for knock-out or knock-in genes in the genome. Considering these restrictions, it is vital to find an accurate and constant method for editing the genomes of crop plants.

A novel editing strategy that serves this purpose is base editing which has been considered a substitute and more proficient tool [47]. It is a simple and accurate method for nucleotide changes deprived of the creation of DNA DSBs [48,49]. The base editing pipeline still relies on the gRNA-guided target finding in the genome, however, involving the inactive CRISPR–Cas9 nuclease (which cannot make DSBs) fused to the deaminase enzyme (cytosine or adenosine) component that manipulates the nucleotide conversion. The cytosine base editors (CBEs) and adenine base editors (ABEs) catalyze the conversion of C to T and A to G (transition base changes), respectively. Since their use in the beginning and later for advanced application with increased editing efficiencies, various generations of CBES and ABEs have evolved. ABEs and CBEs can make single base editing at a time. Therefore, bringing both editing to one platform led to the invention of the dual base editor that can create both C to T and A to T base substitution, expanding the potential of base editing.

Further, to broaden the applicability of the base editing, not just limiting the base editing with transition base conversion, efforts are made to develop the base editors for transversion base substitution. C-to-G Base Editors (CGBE) are created by fusing a Cas9 nickase (nCas9-D10A) to a cytidine deaminase and an uracil-N-glycosylase (UNG). A newly evolved cytidine base-editing tool proposes a valuable substitute [50]. In wheat, acetolactate synthase (ALS) is an ideal herbicide tolerance target gene for base editing that can contain point mutations conferring adequate herbicide tolerance with a minorconsequence to plant productivity [51]. This is why mutations in ALS genes have been done through cytidine base editing in some diploid plant species, including Imidazolinone tolerance in rice [52] and tribenuron tolerance in *Arabidopsis* [53].

Prime editing (PE) is a versatile and accurate gene-editing technology that utilizes nCas9 attached to an engineered reverse transcriptase. It is also known as homology-directed repair (HDR)-independent CRISPR-Cas9 compared to HDR-dependent CRISPR-Cas9. At the same time, template RNA is linked to sgRNA to custom prime editing guidance RNA (pegRNA), which both stipulates the target site and encodes the anticipated editing sequence [54] reports that pairs of pegRNA were capable of exactly deleting 710 bps or accurately replace a sequence of 108 bps. To date, prime editing has been applied to corn, rice, wheat, and tomato [55–57].

3.2. Transgene Free Genome Editing

Gene editing is extensively used across plant species to study and produce the impact of functional mutations in crop improvement. Conversely, the integration of transgene in the genome of plants upraises essential legislative analysis concerning genetically modified organisms [58]. Conventional genome engineering methods need the transfer and combination of DNA cassettes to encode modified parts into the host genome. DNA fragments are generally degenerated but generate detrimental effects [59]. DNA-free genome editing is becoming a novel and fast expanding movement in biological sciences because of its advantages. This technique opened the roads to targeted genome alterations without

conflict with the genome and uplifted the opportunities to produce non-genetically modified organisms [60,61]. However, DNA-free genome editing comes across the same fundamental issues as transformation approaches. Regardless of the broader arsenal of transformation techniques, the RNA and protein delivery methodologies are less developed for plant cells than for animals. Thus, only the biolistic method and protoplast transfection could be used to generate transgene-free genome-edited plants [62]. Protoplasts were the primary tissue successfully targeted for DNA-free gene editing through polyethylene glycol (PEG) mediated fusion. Hence, ribonucleoprotein (RNP) complex or mRNA mix with PEG and combine with the protoplast.

DNA-free genome editing was studied by transfecting guide RNA and Cas9 protein into protoplasts of tobacco, Arabidopsis thaliana, rice, and lettuce and achieved targeted mutagenesis in regenerated plants at frequencies of up to 46% [63]. To achieve DNA-free genome-edited plants, the wheat embryo has been used for particle bombardment using CRISPR/Cas9 RNAs [64].This study achieved highly efficient and precise DNA-free genome editing by a transient expression that produced homozygous mutant plants in the T0 generation. In addition, a recently published report by [65] revealed an efficient method of DNA-free genome editing in potato (*Solanum tuberosum*) protoplast using circular and linearized plasmid DNA fragments that showed high expression of the transgene and upto 95% gene editing events in protoplast derived potato calli [65].

3.3. Multiplexed CRISPR technologies for gene editing

Multiplex genome-editing technologies are versatile and powerful tools for precisely modifying numerous specific DNA loci in the genome. In this technique, various gRNA and Cas9 enzymes are expressed at once [61], facilitating potent bioengineering applications and greatly improving the possibility and genome editing efficiencies [64,66]. These approaches have significantly improved the achievability of desired alterations at multiple nucleotide levels in the target genome. With numerous sgRNA targets, several genomes can be modified concurrently in any crop plant. Using this technique, various traits could be introduced and new plant varieties. Furthermore, numerous individuals from many families could be targeted by combining multiple sgRNAs into a plasmid vector [48,67]. The principal advantage of CRISPR is multiplex genome editing, frequently used to edit multiple sgRNA targets in the genome. It has two methods; in the first method, a single promoter is used to direct the expression of numerous sgRNAs as one transcript. In another case, one promoter is used to express each sgRNA target [68]. In plants, multiplex genetic engineering is primarily centered on traits like herbicide tolerance. Still, in recent times, it has been extended to several parameters such as metabolic engineering, plant development and molecular farming, and hormone biosynthesis and perception with >100 concurrent targeting events [69]. Multiplex genome editing involves concurrently targeting numerous sequences that are closely associated, such as homeoalleles in the plants with polyploidy, multiple alleles of the same gene, or members of the same gene family. Therefore, multiple genomes editing technologies will offer a more speedy way of producing new variations in the varieties of economically important crops.

3.4. Production of Mutant Libraries

The high efficiency of CRISPR-mediated mutagenesis of crop plants allows the improvement of the high-throughput mutagenesis approach. The common use of CRISPR/Cas9 is important for designing mutant libraries to learn the genetic means behind crop improvement. The preparation of mutant libraries is an efficient and promising tool [70]. CRISPR/Cas9 is a potent method for constructing mutant libraries, and its targeting ability could be altered by changing the 18-20 bp target binding order in the sgRNA target. Tomato transformation was carried out by transforming pooled CRISPR libraries to generate a group of mutant lines with the least transformation attempts and, in less time [71]. In this study, a single transformation attempt was performed using the CRISPR

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library that targeted immunity-related leucine-rich repeat subfamily XII genes resulting in inherited mutation retrieving in 15 genes out of the total 54 targeted genes.

Further, to improve productivity, they constructed a second library containing three sgRNAs per construct to target 18 genes, resulting in mutagenesis in 15 out of a total of 18 targeted genes [71]. For the rice plant transformation, mutant libraries were generated with loss-of-function mutation [72]. These plants showed phenotypic changes like lethality and sterility during their cultivation in the field.

4. Impact of CRISPR-Cas9-based gene editing on plant productivity and stress tolerance

Abiotic stress negatively affects plant growth and production. It affects diverse biochemical, morphological, and physiological parameters crucial for plant growth. So far, CRISPR-Cas mediated gene editing is broadly used and adopted in almost 20 agronomically essential crops [73]. Identification of ABA-induced transcription repressors (AITRs) as a new transcription factors family that play an important role as feedback regulators of ABA signaling and loss-of-function of *AITR* genes led to declined ABA sensitivity in *Arabidopsis* [74]. Alternation in the expression of *AITR* genes resulted in abiotic stress tolerance, including drought and salinity in *Arabidopsis* [53].

Conversely, overexpression of the *AITR5* gene showed decreased salinity stress tolerance in *Arabidopsis* [75]. Significantly, knock-out-of-six *AITR* genes showed improved drought and salinity stress tolerance in *Arabidopsis* exclusive of fitness cost [75]. Similarly, knock-out of three *AITR* genes *aitr2*, *aitr5*, and aitr6 (*aitr256*) in *Arabidopsis* plant with triple mutant showed tolerance to drought and salt stress through CRISPR/Cas9 technology which yielded quintuple mutants to increase the tolerant capacity for stress in the plants [76]. In rice, CRISPR/Cas9 mediated knockout of a zinc finger transcription factor and a miRNA, *OsmiR535,* conferred increased tolerance against abiotic stresses in rice plants. In addition, these mutant plants showed higher leaf water retention, enhanced stomatal conductance, root, shoot, and leaf architecture [77,78]. CRISPR-Cas9-based editing of abiotic stresses, including drought, salt, heat, cold, and heavy metal stress-responsive genes and their negative regulators, and mode of plant transformation for development of stress tolerance in plants have been listed in **Tables 1, 2 and 3**. A simplified workflow for CRISPR/Cas9-mediated genome editing in plants has been displayed in **Figure 2**.

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Table 1. Application of the CRISPR-based genome editing approach in plants for improvement of drought and salinity stress tolerance.

Stress Tolerance	Plant species	Target Gene	Gene ID	Method of Delivery	Reference
Drought tolerance	*Arabidopsis thaliana*	*AtOST2*	NM_001335616	Agrobacterium-mediated	[79]
Drought Tolerance	*A. thaliana*	*AtAREB1*	AT1G45249.3	Agrobactrium -mediated	[80]
Drought Tolerance	*A. thaliana*	*AtAVP1*	NM_101437	Agrobacterium-mediated	[81]
Drought Tolerance	*A. thaliana*	*AtmiR169a*	-	Agrobacterium-mediated	[82]
Drought tolerance	*Brassica napus*	*BnaA6.RGA*	LOC106445425	Agrobacterium-mediated	[83]
Drought Tolerance	*Cicer arietinum*	*At4CL, AtRVE7*	LOC101502718, LOC101509066	PEG-mediated	[84]
Drought tolerance	*Glycine max*	*GmMYB118*	GLYMA_17G094400	Agrobacterium-mediated	[85]
Drought tolerance	*Oryza sativa*	*OsERA1*	LOC_Os01g53600	Agrobacterium-mediated	[86]
Drought tolerance	*O. sativa*	*OsSAPK2*	LOC_Os07g42940	Agrobacterium-mediated	[36]
Drought tolerance	*O. sativa*	*OsSRL1, OsSRL2*	LOC_Os01g54390	Agrobacterium-mediated	[87]
Drought Tolerance	*O. sativa*	*OsDST*	LOC_Os03g57240	Agrobacterium-mediated	[59]
Drought Tolerance	*O. sativa*	*OsNAC14*	Os01g0675800	Agrobacterium-mediated	[88]
Drought Tolerance	*O. sativa*	*OsPUB67*	*NP_001065331.1*	Agrobacterium-mediated	[89]
Drought tolerance	*Solanum lycopersicum*	*SlNPR1*	KX198701	Agrobacterium-mediated	[39]
Drought Tolerance	*S. lycopersicum*	*SlMAPK3*	AY261514	Agrobacterium-mediated	[31]
Drought Tolerance	*S. lycopersicum*	*SlLBD40*	Solyc02g085910	Agrobacterium-mediated	[90]
Drought Tolerance	*S. lycopersicum*	*SlARF4*	Solyc11g069190	Agrobacterium-mediated	[75]
Drought tolerance	*Triticum Aestivum*	*TaDREB2, TaDREB3, TaERF3*	DQ353852.1 EF570122.1	PEG-mediated	[91]
Drought Tolerance	*T. Aestivum*	*TaDREB2, TaERF3*	DQ353852.1, EF570122.1	Agrobacterium-mediated	[91]
Drought tolerance	*Zea mays*	*ZmARGOS8*	GQ184457	Agrobacterium-mediated	[38]
Salt tolerance	*A. Thaliana*	*AtWRKY, AtWRKY4*	-	Agrobacterium-mediated	[92]
Salt tolerance	*A. Thaliana*	*AtACQOS*	*AT5G46510*	Agrobacterium-mediated	[93]
Salt tolerance	*Glycine max*	*GmDrb2a, GmDrb2b*	NM_001254313	Agrobacterium-mediated	[94]
Salt tolerance	*G. max*	*GmAITR*	XM_003549793	Agrobacterium-mediated	[95]
Salt tolerance	*Medicago Truncatula*	*MtHEN1*	Medtr4g094545	Agrobacterium-Mediated	[94]
Salt tolerance	*O. sativa*	*OsDST*	LOC_Os03g57240	Agrobacterium-mediated	[77]

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Salt tolerance	*O. sativa*	*OsRAV2*	LOC_Os01g04800	Agrobacterium-mediated	[96]
Salt tolerance	*O. sativa*	*OsRR22*	KF892986	Agrobacterium-mediated	[97]
Salt tolerance	*O. sativa*	*OsNAC45*	KT957809	Agrobacterium-mediated	[98]
Salt tolerance	*O. sativa*	*OsBBS1*	LOC_Os03g24930	Agrobacterium-mediated	[99]
Salt tolerance	*O. sativa*	*OsAGO2*	LOC4336991	Agrobacterium-mediated	[100]
Salt tolerance	*O. sativa*	*OsVDE*	*LOC_Os04g31040*	Agrobacterium-mediated	[4]
Salt tolerance	*O. sativa*	*OsNAC041*	LOC_Os03g013300	Agrobacterium-mediated	[101]
Salt tolerance	*O. sativa*	*OsSAPK2*	LOC_Os07g42940	Agrobacterium-mediated	[36]
Salt tolerance	*O. sativa*	*OsPQT3*	LOC_Os10g29560.1	Agrobacterium-mediated	[102]
Salt tolerance	*O. sativa*	*OsPIL14*	LOC_Os07g05010	Agrobacterium-mediated	[103]
Salt tolerance	*O. sativa*	*OsBGE3*	LOC_Os01g48800	Agrobacterium-mediated	[48]
Salt tolerance	*O. sativa*	*OsSPL10*	LOC_Os06g44860	Agrobacterium-mediated	[104]
Salt tolerance	*O. sativa*	*OsDOF15*	LOC_Os03g55610	Agrobacterium-mediated	[105]
Salt tolerance	*O. sativa*	*OsFLN2*	AP014960	Agrobacterium-mediated	[106]
Salt tolerance	*S. tuberosum*	*StCoilin*	LOC102603469	PEG-mediated	[107]
Salt tolerance	*S. lycopersicum*	*SlHyPRP1*	LOC101257680	PEG-mediated	[108]
Salt tolerance	*T. aestivum*	*TaHAG1*	TraesCS1D02G134200	Agrobacterium-mediated	[109]

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Table 2. Application of the CRISPR-based genome editing approach in plants for improvement of heat and cold stress tolerance.

Stress Tolerance	Plant species	Target Gene	Gene ID	Method of Delivery	Reference
Heat tolerance	*Gossypium hirsutum*	*GhPGF, GhCLA1*	-	Agrobacterium-mediated	[126]
Heat tolerance	*Lactuca sativa*	*LsNCED4*	LOC111879595	Agrobacterium-mediated	[125]
Heat tolerance	*O. sativa*	*OsPDS*	LOC_Os03g08570	Gene gun	[127]
Heat tolerance	*O. sativa*	*OsHSA1*	XM_026023654	Agrobacterium-mediated	[124]
Heat tolerance	*O. sativa*	*OsNAC006*	-	PEG-mediated	[128]
Heat tolerance	*O. sativa*	*OsPYL1/4/6*	-	Agrobacterium Mediated	[129]
Heat tolerance	*S. lycopersicum*	*SIAGL6*	Solyc01g093960	Agrobacterium-mediated	[121]
Heat tolerance	*S. lycopersicum*	*SlCPK28*	Solyc02g083850	Agrobacterium-mediated	[130]
Heat tolerance	*S. lycopersicum*	*SlMAPK3*	NM_001247431.2	Agrobacterium-mediated	[122]
Heat tolerance	*S. lycopersicum*	*SlBZR1*	Solyc04g079980	Agrobacterium-mediated	[123]
Heat tolerance	*Z. mays*	*ZmTMS5 gene*	-	particle bombardment	[46]
Cold tolerance	*A. thaliana*	*AtCBF1, AtCBF2*	AT4G25490, AT4G25470	Agrobacterium-mediated	[53]
Cold tolerance	*O. sativa*	*OsAnn3*	LOC_Os07g46550	Agrobacterium-mediated	[131]
Cold tolerance	*O. sativa*	*OsPIN5b, GS3, Os-MYB30*	Os08g0529000, Os03g0407400, Os02g0624300	Agrobacterium-mediated	[132]
Cold tolerance	*O. sativa*	*OsAnn5*	-	Agrobacterium-mediated	[131]
Cold tolerance	*O. sativa*	*OsPRP1*	AB055842	Agrobacterium-mediated	[133]
Cold tolerance	*S. lycopersicum*	*SlCBF1*	-	Agrobacterium-mediated	[134]

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Table 3. Application of the CRISPR-based genome editing approach in plants for improvement of metals and herbicide stress tolerance.

Stress Tolerance	Plant species	Target Gene	Gene ID	Method of Delivery	Reference
Metal stress tolerance	*A. thaliana*	*Atoxp1*	At5G37830	Agrobacterium-mediated	[146]
Metal stress tolerance	*O. sativa*	*OsARM1*	Os05g37060	Agrobacterium-mediated	[150]
Metal stress tolerance	*O. sativa*	*OsNramp5*	Os07g0257200	Agrobacterium-mediated	[151]
Metal stress tolerance	*O. sativa*	*OsLCT1*	AB905363	Agrobacterium-mediated	[70]
Metal stress tolerance	*O. sativa*	*OsHAK1*	Os04g32920	Agrobacterium-Mediated	[149]
Metal stress tolerance	*O. sativa*	*OsPRX2*	Os02g053770	Agrobacterium-Mediated	[60]
Metal stress tolerance	*O. sativa*	OsATX1	-	Agrobacterium-Mediated	[152]
Herbicide resistance	*B. napus*	*BnALS*	LOC106353716	Agrobacterium-Mediated	[83]
Herbicide resistance	*Manihot esculenta*	*MeEPSPS*	Manes.05G046900	Agrobacterium-Mediated	[153]
Herbicide resistance	*O. sativa*	*OsALS*	LOC4329938	Agrobacterium-Mediated	[154]
Herbicide resistance	*O. sativa*	*OsALS*	MN268687	Agrobacterium-Mediated	[155]
Herbicide resistance	*O. sativa*	*OsTB1*	AF322143	Agrobacterium-Mediated	[156]
Herbicide resistance	*O. sativa*	*OsPUT1/2/3*	Os02g0700500, Os12g0580400,Os03g0576900	Agrobacterium-Mediated	[157]
Herbicide resistance	*O. sativa*	*OsACC*	LOC_Os05g22940	Agrobacterium-Mediated	[43]
Herbicide resistance	*O. sativa*	*OsEPSPS*	AF413081	PEG-mediated	[158]
Herbicide resistance	*O. sativa*	*OsEPSPS*	AF413081	biolistic gene transfer	[158]
Herbicide resistance	*O. sativa*	*OsALS-1, OsALS-2, OsALS-3,OsALS-4*	-	Agrobacterium-Mediated	[159]
Herbicide resistance	*Saccharum officinarum*	*SoALS*	MZ268741	biolistic gene transfer	[30]
Herbicide resistance	*S. lycopersicum*	*SlEPSPS*	Solyc01g091190	Agrobacterium-Mediated	[160]
Herbicide resistance	*S. lycopersicum*	*SlALS1, SlALS2,*	Solyc06g059880, Solyc03g044330	Agrobacterium-Mediated	[160]
Herbicide resistance	*S. lycopersicum*	*Slpds1*	Solyc03g123760	Agrobacterium-Mediated	[160]
Herbicide resistance	*T. aestivum*	*TaALS*	TraesCS6A02G288000	Biolistic-mediated	[97]
Herbicide resistance	*Z. mays*	*ZmALS1, ZmALS2*	LOC100381801, LOC100274341	Agrobacterium-Mediated	[161]
Herbicide resistance	*Z. mays*	*MS26*	LOC100191749	Biolistic-mediated	[161]

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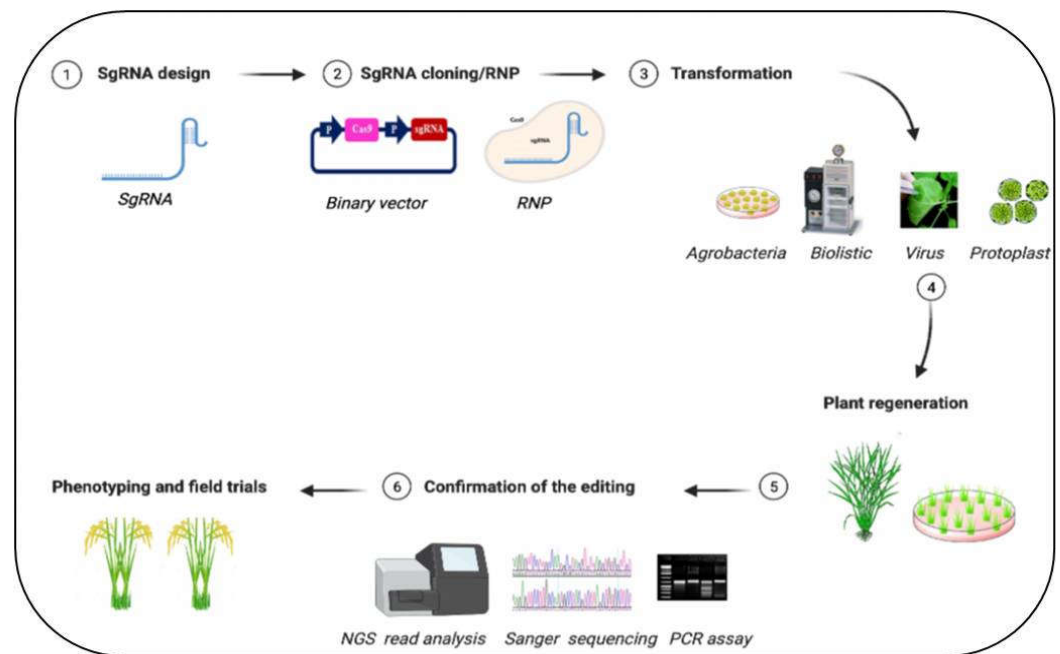

Figure 2. Simplified workflow for CRISPR/Cas9-mediated plant genome editing. The generation of edited plants with thedesired phenotype starts with the design of (1) guide RNA (gRNA) for a specific target sequence and (2) cloning of the sequence to express the sgRNA into a binary vector containing the Cas DNA sequence or forming ribonucleic protein complex (RNP). Then the (3) delivery of CRISPR-Cas materials into the plant tissues through various methods, (4) regeneration of the transgenic plants followed by (5) assays to confirm the editing events with (6) improved trait of crop plants. Figure created with BioRender.com (https://app.biorender.com/biorender-templates)—accessed on 25 May 2022.

4.1. Drought stress tolerance

Overexpression of several drought-responsive genes and transcription factors increases the accumulation of signaling molecules and metabolic compounds and enhance drought tolerance in plants [77,110,111]. The expressions of drought-sensitive (S) genes enhance susceptibility in plants to drought through hormonal disproportion, declined antioxidant activities, and increasing reactive oxygen species (ROS) production. Over-expression of AREB1 has shown improved tolerance to drought stress, whereas the *AREB1* knock-out mutant showed higher sensitivity to drought stress [112]. CRISPR/Cas9 targeted mutagenesis of *SlLBD40*, a lateral organ boundaries domain transcription factor that enhances drought tolerance in tomatoes compared with overexpressing transgenic and WT tomato plants; knockout of *SlLBD40* by CRISPR/Cas9 enhanced the drought tolerance of tomato [90]. CRISPR-Cas9 edited tomato (*Solanum lycopersicum*) mutant plants knock-out for *SlMAPK3* gene showed enhanced drought stress response [113]. Under drought, these mutant plants exhibited severe wilting symptoms, elevated levels of H_2O_2, reduced antioxidants, and increased membrane damage. These results substantiate that *SlMAPK3* is implicated in drought stress response in tomato plants by protecting the cell membrane. Knockout of tomato *Auxin Response Factor* (*SlARF4)* gene improves tomato resistance to water deficit [75]. Improved drought stress tolerance was observed in Arabidopsis by CRISPR/dCas9 fusion with a *Histone Acetyl Transferase* (*AtHAT*) gene [80]. CRISPR-Cas9-based editing of *pathogenesis-related 1* (*NPR1*) gene in tomato exhibited drought response [39] by enhancing stomatal aperture, malondialdehyde (MDA) level, H_2O_2 content and ion leakage. However, the level of antioxidant activities was found declined than WT plants. The *SlNPR1* plays a significant role in directing responses against drought stress in tomatoes and other crop plants. Multiple *SlNPR1* variants can be developed through gene editing to enhance drought tolerance in a wide range [39].Drought-induced SINA protein 1 (*OsDIS1*), drought and salt-tolerant protein 1 (*OsDST*), and ring finger protein 1 (*OsSRFP1*) genes are negative regulators of drought tolerance. Silencing

these drought-responsive genes improved levels of antioxidant enzymes, decreased concentrations of H_2O_2, and increased tolerance to drought stress in rice plants [77,110]. *Enhanced Response 1 (ERA1*) protein gene regulates ABA signaling and dehydration responses in plants. In rice, genome editing of *OsERA1* gene enhanced response to drought stress. The mutant plant showed increased sensitivity to ABA and stomatal closure under drought condition [61]. *OsSAPK2* also has the role for ABA-mediated stress tolerance in rice and was confirmed by developing mutants using CRISPR-Cas9 with loss of function mutation. The mutants produced exhibited more drought sensitivity compared to WT plants [36]. Enhanced stomatal response was facilitated by the CRISPR Cas9-induced mutations in the gene encoding *OPEN STOMATA 2 (AtOST2)* in Arabidopsis mutants than WT [79]. Interestingly the *AtOST2* mutants had a high degree of stomatal closure [64]. In rice, *OsSRL1* and *OsSRL2* gene encodes leaf tissue phenotype. The genome-modified lines having homozygous *SRL1* and *SRL2* mutant were found retardation in various characteristics such as the stomata number, stomatal conductance, transpiration rate, chlorophyll content, vascular bundles and other agronomic traits in comparison to wild-type one [87]. Drought tolerance can be obtained through CRSIPR-Cas9 based genome editing by targeting negative regulators or drought-sensitive genes. CRISPR-Cas9-based gene editing in *Zea mays* was carried out to enhance the expression level of the *ARGOS8* gene, which negatively regulates ethylene response, for the development of drought tolerance. Such mutant plants showed improved grain yields in the field under drought stress conditions [114]. WRKY transcription factors regulate the plant's growth and development and involve biotic and abiotic stresses. In plants, *WRKY3* and *WRKY4* genes play an important role in regulating defense response to drought stress [92].

4.2. Salinity stress tolerance

By the year 2050, more than 50% of agricultural lands may get critically salinized [4]. In plants, salt stress causes various physiological and morphological changes because of alterations in the expression of genes and signalling pathways [115]. The key detrimental effects of salinity stress are necrosis, untimely death of old leaves, and harsh interruption of ions in cells [116]. Several genes have been identified and characterized through CRISPR/Cas-based gene editing to improve plant salt tolerance. Knockout of *AtWRKY3* and *AtWRKY4* genes in *A. thaliana* plants using CRISPR-Cas9 exhibited significant upregulation of genes under salt and Me-JA stresses. Such double mutant plants showed sensitivity features to salinity and Me-JA, such as elevation in ion leakage and reduction in antioxidant activities, including peroxidase (POD), catalase (CAT), and superoxide dismutase (SOD)[75][Table 1]. Also the importance of *Acquired Osmotolerance* (*AtACQOS*) gene provided tolerance against salt stress in Arabidopsis is characterized by CRISPR-generated mutants [93]. CRISPR-cas9-based knock-out mutants of abscisic acid (ABA)-induced transcription repressors (AITRs) genes conferred salt stress tolerance in soybean (*Glycine max*) plant [95]. These mutant plants showed increased ABA sensitivity and produced longer roots and shoots than WT plants. Similarly, mutants of *GmDrb2a* and *GmDrb2b* genes showed enhanced salinity stress tolerance in *G. max*[94]. CRISPR-Cas9 mediated editing of *OsRAV2* gene expression was induced by the regulatory function of the GT-1 element in rice and showed tolerance to salt stress [96]. CRISPR mutants with loss of function of *SnRK2* and osmotic stress/ABA-activated protein kinases *SAPK-1* and-*SAPK-2* genes showed resistance to salt stress in rice [36]. In several other studies, the development of CRISPR-mutants in rice to develop salt stress tolerance plants through knock-out of *OsDST* [77], *OsNAC45* [98], *AGO2* (*ARGONAUTE2*) [100], Rice type-B response regulator (*OsRR22*) [97], and *OsBBS1* (bilateral blade Senescenc1) [99] have been carried out. Mutants of *TaHAG1* gene of wheat plants generated through CRISPR-Cas9 showed enhanced salt tolerance [109].

Violaxanthin de-epoxidase (VDE) plays a critical role in plants' abscisic acid (ABA) biosynthesis, growth and stress responses. In rice, the functional benefits of *OsVDE* in salt tolerance are validated. Gene-editing targeting *OsVDE* loci in overexpressed transgenic

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rice was found to have a higher ABA level, stomatal closure percentage and survival rate than the wild type under seedling stage salt stress. [4]. Several plant transcription factor family genes are involved in the salt stress response. NAC transcription factor coding gene, *OsNAC041* confirmed its importance for germinating seeds under salt stress. *Osnac041* mutant obtained by CRISPR/Cas9 method showed increased salt sensitivity compared to the wild plants [101]. Transcription factor *OsDOF15* positively regulates primary root elongation by regulating cell proliferation in the root meristem via restricting ethylene biosynthesis. Loss-of-function of *OsDOF15* impaired primary root elongation and cell proliferation in the root meristem [105]. Some instances where editing on the negative regulator for salt stress tolerance are *OsPQT3* and the DELLA protein SLENDER RICE1 (SLR1). In rice, *OsPQT3* knockout mutants displayed enhanced resistance to oxidative and salt stress with elevated expression of *OsGPX1, OsAPX1* and *OsSOD1* under salt stress [102], and the loss of function of SLR1 promotes mesocotyl and root growth, specifically in the dark and under salt stress [103].

4.3. Heat stress tolerance

High temperature or heat stress is one of the major abiotic stresses that become a severe problem in agricultural production in several regions of the world that causes global warming [117].Plants react to heat stress by activating complex molecular networks, including heat stress-responsive gene expression, signal transduction, and metabolites production. With the advancements in functional and structural genomics techniques in plants, various heat stress-associated genes have been identified and characterized to enhance heat tolerance with advanced biotechnological tools. The Heat shock proteins (HSPs) and heat shock transcription factors (HSFs) are crucial gears and function through the heat stress-response signal transduction pathway, which is linked to ROS accumulation [118]. Hence, heat stress tolerance can be improved by enhancing the ability of plants to detoxify ROS components [119]. This indicated that enhanced tolerance could improve crop plants' antioxidant activities. Heat-induced gene expression and metabolite biosynthesis significantly enhanced heat tolerance in plants. Among all the genome-editing approaches, CRISPR-Cas9 is a revolutionary technique for genome editing in a precise manner to learn the molecular pathways associated with heat stress and improve crop heat tolerance [96]. Tomato (*Solanum lycopersicum*) is considered an ultimate model to test CRISPR/Cas9-based gene editing because it can endure competent transformation to grain quality enhancement [120]. Currently, CRISPR/Cas9-based genome editing of the heat-sensitive gene, *SlAGAMOUS-LIKE 6* (*SlAGL6*), in tomatoes was generated for heat tolerance, enhancing fruit setting under heat stress conditions [121]. In tomato, the *SlMAPK3* gene belongs to the mitogen-activated protein kinase family and participates in response to diverse environmental stress functions. CRISPR/Cas9-mediated gene editing resulted in *slmapk3* mutants showing enhancedthermo-tolerance compared to WT plants and implying its role as a negative regulator of thermo-tolerance [122]. BRZ1 positively regulates ROS production in the apoplastic region in tomatoes and serves as a component for heat tolerance. This has been validated from the CRISPR-Cas9-based *bzr1* mutants that showed impaired H_2O_2 production in apoplast and heat tolerance by declined Respiratory Burst Oxidase Homolog 1(RBOH1)[123]. Development of CRISPR/Cas-mediated HSA1 (heat-stress sensitive albino 1) mutants of tomato showed increased sensitivity to heat stress compared to wild-type plants [124]. In maize, CRISPR mutants of the *thermosensitive genic malesterile 5 (TMS5)* gene improved thermosensitive male-sterile plants [46]. In lettuce, the germination of the seeds at a higher temperature was achieved through knockouts of *NCED4*, a key regulatory enzyme in the biosynthesis of abscisic acid (ABA). Therefore mutants of *LsNCED4* could be commercially valuable in production areas with high temperatures [125].

4.4. Cold stress tolerance

Low temperature is a key abiotic stressor that adversely influences plant growth and productivity. In plants, cold stress tolerance is a highly intricate trait concerning several diverse cell compartments and metabolic pathways [135]. Conventional breeding approaches have achieved adequate success in enhancing the cold tolerance of significant crop plants relating to inter-specific or inter-generic hybridization. Cold stress causes damaged seedlings, poor growth, and a low germination rate in rice. It can also decrease grain yield at reproductive phage in rice [136,137]. CRISPR/Cas9 is an attractive and accessible technology for developing non-transgenic genome-edited crop plants to overcome climate change and ensure future food security [103]. In rice, editing is guided to knockout some of the negative regulator transcription factors to increase plant tolerance for cold. OsMYB30 is a transcription factor that binds to the promoter of the β-amylase gene and negatively influences cold tolerance. Under cold stress, OsMYB30 makes a complex with OsJAZ9 and inhibits the expression of β-amylase gene, thus affecting starch degradation and maltose accumulation which may contribute to increasing cold sensitivity [138]. CRISPR-Cas9-based gene editing of three genes, *OsPIN5b*, *GS3*, and *OsMYB30*, mutated simultaneously, showed enhanced yield and tolerance to cold stress [132]. Plant annexins are involved in the regulation of plant development and protection from environmental stresses: Rice annexin genes *OsAnn3 and OsAnn5* are positive regulators of cold stress tolerance at the seedling stage. The Knocking out of *OsAnn3 and OsAnn5* resulted in sensitivity to cold treatments [131]. Also in rice, *OsPRP1* enhances cold tolerance by modulating antioxidants and maintaining cross-talk through signaling pathways. Knockout of *OsPRP1* induced cold sensitivity in rice, and mutant lines accumulated less antioxidant enzyme activity and lower levels of proline, chlorophyll, abscisic acid (ABA), and ascorbic acid (AsA) content relative to WT under low-temperature. Tomato plants are sensitive to chilling stress; therefore, their fruits are more prone to get damaged by cold stress. CRISPR-Cas9 based *cbf1* mutants showed that C-repeat binding factor 1 (CBF1) protects the tomato plant against chilling/cold damage and decreases electrolyte leakage [139]. These plants also showed a higher accumulation of hydrogen peroxide and indole acetic acid, thus, providing tolerance to cold stress in tomato plants. The expression of ten transcription factors from the WRKY family was observed two-fold higher under cold stress [140]. In Cucumber, over-expression of the *CsWRKY6* gene showed enhanced tolerance to cold stress and sensitivity to ABA and proline accumulation [141]. RNA sequencing of *Brassica napus* revealed various genes from the WRKY family that play an important role in cold response [142].

4.5. Metal stress tolerance

Heavy metal stress is one of the key problems that adversely affect the agricultural productivity of plants. Plants practice oxidative stress upon contact with heavy metals, leading to cellular injury [143]. Additionally, the accumulation of metal ions in plants perturbs cellular ionic homeostasis. Therefore, plants have developed detoxification mechanisms to reduce heavy metal exposure's damaging effects and accumulation. Such mechanisms involve controlled elimination of toxic ions from roots, metal uptake, efficient neutralization of metal ions in the protoplast, and appropriation or translocation to remote organs [144]. Various genes direct these mechanisms to enhance tolerance to heavy metal stress [145]. For example, the loss-of-function mutant of γ-glutamylcyclotransferase showed defensive characteristics against heavy metal toxicity [112, suggesting that the loss-of-function mutants of OXP1 and γ-glutamylcyclotransferase demonstrate heavy metal and xenobiotic detoxification due to increased glutathione (GSH) accumulation. Therefore, developing CRISPR-Cas9 mediated mutants of plants would be useful to fight against the heavy metal stress in plants. Recently, Baeg et al. [146] developed oxp1/CRISPR mutant *Arabidopsis* plants that showed resistance to Cd, suggesting an improved capability of heavy metal detoxification in mutant plants compared to WT Col0 plants. Consequently, this study showed a way to confer resistance to xenobiotics and heavy metals in plants by indel mutations using the gene-editing method [146].

In rice, the roots absorb Cd from the soil with the transporters *OsNramp1*, *OsNramp5*, and *OsCd1*. *OsHMA3* does the role of Cd sequestration into root vacuole and negatively regulates xylem loading, and *OsLCT1* is involved in Cd transport to the grains [147]. Manipulating the expression of these transporter genes by genome editing has found some success in reducing Cd in the grain crop. The CRISPR-Cas9-based mutants of *OsNramp5 andOsLCT1* genes resulted in a low Cd level in rice. [70,113,148]. Similarly, *OsARM1* regulates As-associated transporter genes in rice. It is expressed in the phloem of the vascular bundle in basal and upper nodes. Knock-out of the *OSARM1* by CRISPR improves tolerance, while its overexpression has increased sensitivity to As [146]. Cs+-permeable *OsHAK1* transporter in rice is the major pathway for Cs+ uptake and translocation. To minimize the radioactive caesium (Cs) uptake by rice plants in Fukusima soil contaminated with 137 Cs+, the CRISPR-Cas system was used to obtain transgenic plants lacking *OsHAK1* function. The *OsHAK1* knock-out plants displayed strikingly reduced levels of 137 Cs+ in roots and reduced radioactive caesium contents [149]. Another instance of using the crisper-cas-based editing in rice is to know the function of a potential target *OsPRX2* for improved potassium deficiency tolerance. OsPRX2 is known to reduce the production of ROS in a K+ limiting condition. It was found overexpression of *OsPRX2* causes the stomatal closing and K+ deficiency tolerance to increase, while knockout of *OsPRX2* leads to serious defects in leaves phenotype and the stomatal opening under the K+-deficiency tolerance [60].

4.6. Herbicide stress tolerance

Weed is the major global agricultural constraint that limits crop production by challenging crop plants for nutrients, soil moisture, light, space, and CO2. Its growth is one of the key factors that influence the quality and yield of crop plants [162]. Several approaches have been tried to eradicate weeds [163]. The herbicide application is the key tool used for weed management in recent crop production systems [164]. Herbicide tolerance is one of the most important traits of crop plants that advance farming techniques and productivity of crop plants. CRISPR-Cas9-based gene editing to develop herbicide-resistant crop plants is now the ideal system to control weeds [165]. Herbicide-tolerant crop plants showed higher yield and could minimize toxicity to the environment and our body three times compared to crops cultivated through the conventional method [166]. This should be adapted as an important practice for high-scale farming; cost-effective and requires less effort developed DNA-free wheat germplasms containing herbicide tolerance mutations that provide tolerance to aryloxyphenoxy propionate-, sulfonylurea-, and imidazolinone-type herbicides by base editing the acetyl-coenzyme A carboxylase and *acetolactate synthase (ALS)* genes [98]. *Acetolactate synthase 1* (*ALS1*) is one of crop plants' most important enzymes responsible for herbicide tolerance. CRISPR-mediated gene editing technique has also been applied to introduce herbicide tolerance in crop plants [Table 3]. A new herbicide tolerance trait has been incorporated in oryza sativa through CRISPR-based gene editing of the *OsALS1* gene [155,167]. Mutants of rice generated by developing a new allele (G628W) by G-to-T transversion at 1882 positon in *OsALS* gene showed strong herbicide tolerance. The progenies of rice mutants were transgene-free and harbouring homozygous allele (G628W) that were agronomically similar to the wilting type. These mutant plants of rice conferred resistance to imazethapyr (IMT) and imazapic (IMP) herbicides [155]. CRISPR-Cas9 mediated gene editing can be useful to generate herbicide-tolerant crop plants. The CRISPR-Cas9-based targeted mutagenesis of three genes ALS (acetolactate synthase), EPSPS (5-Enolpyruvylshikimate-3-phosphate synthase), and pds (phytoene desaturase) conferred herbicide resistance in *Solanum lycopersicum* cv. Micro-Tom [160]. These herbicide tolerance traits offer a potentially powerful approach to weed management. Thus, the CRISPR-based genome editing tool could precisely advance the engineering of herbicide-resistant genes in crop plants.

Genome-editing approaches could demonstrate plant tolerance to abiotic stresses by targeting stress-responsive genes, sensitive genes or negatively regulating genes that

control abiotic stress responses. Additionally, the expression of sensitive genes enhance abiotic stress responses in plants through impaired biochemical (chlorophyll content, changes in antioxidants activities, increased ROS production, ion leakage, lipid peroxidation), physiological (reduced biomass, photosynthetic rate, and higher transpiration rates) and phenotypic (flowers/pods abortion) responses that results in reduced crop yield. Remarkably, CRISPR-Cas9 mediated gene editing approach offers better stress resilience to crop plants though destruction/ modification in the target protein that resulted in modulating these biochemical, physiological, and morphological parameters. Furthermore, these genetically edited CRISPR plants show elevated photosynthetic capacity, increased root length and density, increased biomass, increased nutrient accessibility, stomatal closure, higher chlorophyll content, reduced transpiration rate, the structural adaptation of membranes, and increased RWC, decreased EL and MDA content, reduced metal accumulation that results in abiotic stresses tolerance in plants **Figure 3**.

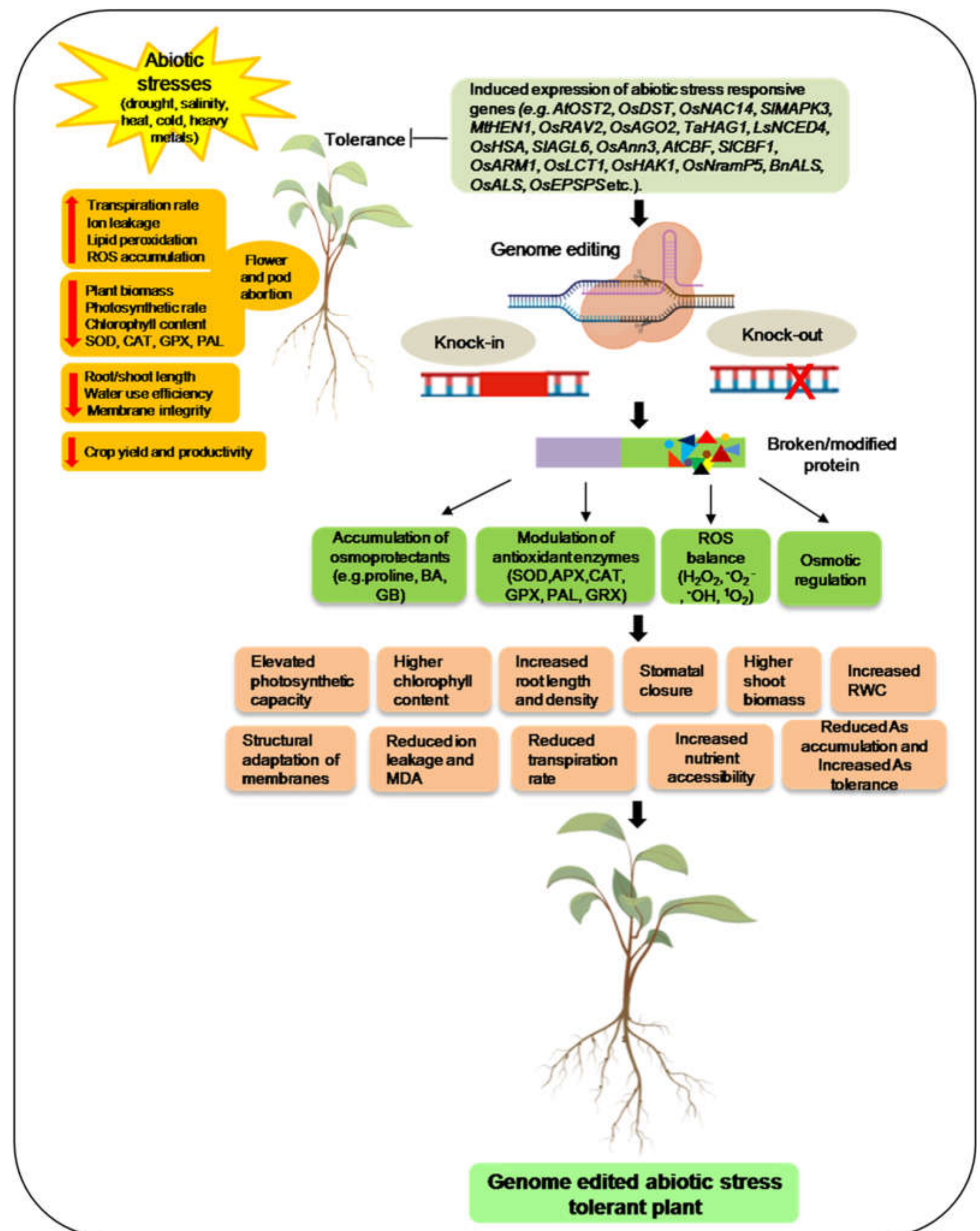

Figure 3. Schematic representation of genome editing mediated abiotic stress (drought, salinity, heat, cold, heavy metals) tolerance in plants. The model shows stress-induced expression of abiotic

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stress responsive gene that lead to reduced plant biomass; photosynthetic rate; SOD, CAT, GPX, and PAL activities; and chlorophyll content and increased reactive oxygen species (ROS), flower and pod abortion, transpiration rate, ion leakage, and lipid peroxidation. Genome edited knock-out/knock-in of stress responsive genes resulted in broken/ modified protein that modulates biochemical and physiological characteristics in plants and provide abiotic stress tolerance. SOD, superoxide dismutase; CAT, catalase; GPX, guaiacol peroxidase; PAL, phenylalanine ammonia-lyase; MDA, malondialdehyde; RWC, relative water content; EL, electrolytic leakage, As, Arsenic. Figure created with BioRender.com (https://app.biorender.com/biorender-templates.

5. Advantages of genome editing approach over breeding and transgenic technologies

The conventional breeding procedure involves cross breeding to introduce useful traits from a donor line to elite recipient lines. Similarly, mutation breeding is carried out to introduce random mutation genome-wide that greatly expands the genomic diversity. However, this procedure requires a lengthy period (8-12 years) as there is a need for repeated backcrossing to the recipient background to ensure the transfer of the desired trait only. Useful genes or traits can also be transferred from other organisms using transgenic breeding, but it involves random integration of the foreign DNA in the genome. Therefore, the development of any transgenic line will have to pass through the lengthy and costly process of regulatory evaluation before its commercialization. Genome-editing technology has a potential advantage compared to all these methods. Genome editing can make small precise changes in a plant's existing DNA that mimic changes that could occur naturally. It can efficiently modify the plant genome for trait improvement and does not require foreign DNA integration. Repeated backcrossing is not required in this case, and transgene-free lines can be ready in less time (2-5 years). So essential characteristics such as better resilience to climate extremes could be made available more rapidly helping to ensure a secure food supply. Recently, countries such as the USA, China, India, the UK, and many others have allowed genome-edited plants to undergo a different regulatory process than those applied to genetically engineered products. One of the advantages of CRISPR tools over other genome-editing technologies is its potential for multiplexing, the simultaneous editing of multiple target sites [168]. Gene editing has several advantages over previous technologies, most meaningfully allowing for targeted, single gene mutation throughout the whole plant genome. The CRISPR technology of new plant breeding tools deals with an easier, more adaptable, and precise form of mutagenesis that enables the transfer of the anticipated trait to progeny deprived of any efficacy [169]. This method can execute mutations to an exact site inside the targeted gene, making the plants' properties important [170] as it can be automated to target specific segments of genetic code or edit DNA with better accurateness [171].

7. Conclusion and future perspectives

CRISPR/Cas9 is considered the method of choice to edit the genome over other genome editing techniques such as ZFNs and TALENs for its high efficiency, low cost, and ease of use. It has been used to modify a wide range of plant species to make sequence-specific editing to characterize the function of genes and their ultimate use for trait improvement [172]. It can induce editing in many sites in the genome with the use of multiple gRNAs. This is helpful to stack multiple traits in an elite variety [48] and target multiple members in multiple gene families [173]. Not just limited to editing, this system uses site-specific modification in the genome, such as epigenetic changes [174], regulation of gene expression [151] and base editing. This is done with the fusion of the effector protein of dead' Cas9 (dCas9) protein which is catalytically dead but has the DNA binding activity. In this way, the fusion protein is guided to reach specific sites in the genome to do its job [175]. The CRISPR interfering system (CRISPRi) can potentially produce effective and precise transcriptional control without editing [176]. So, this is better than the RNAi technology. This is again carried with the binding of SgRNA to dCas9. The binding of the SgRNA to the complementary region blocks the transcriptional elongation by RNA polymerase, expressing the gene without undergoing cell death and damage to the genome

[177]. CRISPR/Cas system can also accomplish gene replacement in plants through targeted integration of specific genes through homology-mediated recombination. Moreover, the CRISPR/Cas can recombine the genome after the DSB in a heterozygous system. This can be used to induce local recombination in the part of the chromosome that does not participate in meiotic recombination, such as the telomeric end and centromeric region, to explore the untapped genetic potential and to narrow down beneficial QTL to the causal allele for precise mapping and gene identification [178,179]

Although significant progress has been made to increase its efficiency and target specificity, more interventions are required to make it a further powerful tool. Few such areas include introducing the smaller-sized CRISPR system for efficient genome editing. The existing CRISPR/Cas9 is relatively large to pack into viral vectors. Similarly, the requirement of the NGG PAM site for CRISPR cannot address the editing to all locations in the genome. Hence, a multiple PAM site selection will increase the scope of the editing. The transformation rate and editing efficiency using Agrobacterium-based methods are preferred to produce transgenic events; however, not all crops and other plant species respond well to the transformation and regeneration under selection. In addition, to make it transgene-free, the process takes longer to eliminate the transgene by several back-crossings of the plant having the editing. Tissue culture-free-based methods such as RNP, viral delivery and nanoparticle-mediated delivery provide alternative strategies to accelerate the process. It will be less expensive, easy, and reduces the time to generate an edited plant. RNPs do the editing without any footprint in the genome and are considered transgene-free. Nanoparticle-based delivery isnow available for the plasmid, and efforts are being made to load and deliver the RNPs. After the DSB in the genome, the NHEJ repair occurs at high frequency while the HDR is a rare event; however, HDR is required for various applications such as recombining the genome, etc. Therefore, efforts should also be taken to find any protein that enhances HDR and should be guided to DSB sites in fusion with CAS protein.

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Reference

1. Schmidhuber, J.; Tubiello, F.N. Global Food Security under Climate Change. *Proc Natl Acad Sci U S A* 2007, *104*.
2. Boyer, J.S. Plant Productivity and Environment. *Science (1979)***1982**, *218*, doi:10.1126/science.218.4571.443.
3. Pandey, P.; Irulappan, V.; Bagavathiannan, M. v.; Senthil-Kumar, M. Impact of Combined Abiotic and Biotic Stresses on Plant Growth and Avenues for Crop Improvement by Exploiting Physio-Morphological Traits. *Frontiers in Plant Science* 2017, *8*.
4. Wang, W.; Vinocur, B.; Altman, A. Plant Responses to Drought, Salinity and Extreme Temperatures: Towards Genetic Engineering for Stress Tolerance. *Planta* 2003, *218*.
5. Alexandratos, Nikos & Bruinsma, J. WORLD AGRICULTURE TOWARDS 2030 / 2050 The 2012 Revision PROOF COPY. *ESA Working paper* 2012, *12*.
6. Ray, D.K.; Mueller, N.D.; West, P.C.; Foley, J.A. Yield Trends Are Insufficient to Double Global Crop Production by 2050. *PLoS ONE***2013**, *8*, doi:10.1371/journal.pone.0066428.
7. Manavalan, L.P.; Guttikonda, S.K.; Phan Tran, L.S.; Nguyen, H.T. Physiological and Molecular Approaches to Improve Drought Resistance in Soybean. *Plant and Cell Physiology* 2009, *50*.

8. Driedonks, N.; Rieu, I.; Vriezen, W.H. Breeding for Plant Heat Tolerance at Vegetative and Reproductive Stages. *Plant Reproduction* 2016, *29*.
9. Puchta, H.; Dujon, B.; Hohn, B. Homologous Recombination in Plant Cells Is Enhanced by in Vivo Induction of Double Strand Breaks into DNA by a Site-Specific Endonuclease. *Nucleic Acids Research***1993**, *21*, doi:10.1093/nar/21.22.5034.
10. Zhang, Y.; Zhang, F.; Li, X.; Baller, J.A.; Qi, Y.; Starker, C.G.; Bogdanove, A.J.; Voytas, D.F. Transcription Activator-like Effector Nucleases Enable Efficient Plant Genome Engineering. *Plant Physiology***2013**, *161*, doi:10.1104/pp.112.205179.
11. Zhang, F.; Maeder, M.L.; Unger-Wallaced, E.; Hoshaw, J.P.; Reyon, D.; Christian, M.; Li, X.; Pierick, C.J.; Dobbs, D.; Peterson, T.; et al. High Frequency Targeted Mutagenesis in Arabidopsis Thaliana Using Zinc Finger Nucleases. *Proc Natl Acad Sci U S A***2010**, *107*, doi:10.1073/pnas.0914991107.
12. Jiang, W.; Zhou, H.; Bi, H.; Fromm, M.; Yang, B.; Weeks, D.P. Demonstration of CRISPR/Cas9/SgRNA-Mediated Targeted Gene Modification in Arabidopsis, Tobacco, Sorghum and Rice. *Nucleic Acids Research***2013**, *41*, doi:10.1093/nar/gkt780.
13. Chen, K.; Wang, Y.; Zhang, R.; Zhang, H.; Gao, C. CRISPR/Cas Genome Editing and Precision Plant Breeding in Agriculture. *Annual Review of Plant Biology* 2019, *70*.
14. Boyaval, P.; Moineau, S.; Romero, D. a; Horvath, P. Against Viruses in Prokaryotes. *Science (1979)***2007**, *315*.
15. Waltz, E. GABA-Enriched Tomato Is First CRISPR-Edited Food to Enter Market. *Nat Biotechnol* 2022, *40*.
16. Li, J.F.; Norville, J.E.; Aach, J.; McCormack, M.; Zhang, D.; Bush, J.; Church, G.M.; Sheen, J. Multiplex and Homologous Recombination-Mediated Genome Editing in Arabidopsis and Nicotiana Benthamiana Using Guide RNA and Cas9. *Nature Biotechnology* 2013, *31*.
17. Donovan, S.; Mao, Y.; Orr, D.J.; Carmo-Silva, E.; McCormick, A.J. CRISPR-Cas9-Mediated Mutagenesis of the Rubisco Small Subunit Family in Nicotiana Tabacum. *Frontiers in Genome Editing***2020**, *2*, doi:10.3389/fgeed.2020.605614.
18. Kumar, M.; Ayzenshtat, D.; Marko, A.; Bocobza, S. Optimization of T-DNA Configuration with UBIQUITIN10 Promoters and TRNA–SgRNA Complexes Promotes Highly Efficient Genome Editing in Allotetraploid Tobacco. *Plant Cell Reports***2022**, *41*, doi:10.1007/s00299-021-02796-0.
19. Nekrasov, V.; Staskawicz, B.; Weigel, D.; Jones, J.D.G.; Kamoun, S. Targeted Mutagenesis in the Model Plant Nicotiana Benthamiana Using Cas9 RNA-Guided Endonuclease. *Nature Biotechnology* 2013, *31*.
20. Shan, Q.; Wang, Y.; Li, J.; Zhang, Y.; Chen, K.; Liang, Z.; Zhang, K.; Liu, J.; Xi, J.J.; Qiu, J.L.; et al. Targeted Genome Modification of Crop Plants Using a CRISPR-Cas System. *Nature Biotechnology* 2013, *31*.
21. Liang, Z.; Zhang, K.; Chen, K.; Gao, C. Targeted Mutagenesis in Zea Mays Using TALENs and the CRISPR/Cas System. *Journal of Genetics and Genomics***2014**, *41*, doi:10.1016/j.jgg.2013.12.001.
22. Miao, J.; Guo, D.; Zhang, J.; Huang, Q.; Qin, G.; Zhang, X.; Wan, J.; Gu, H.; Qu, L.J. Targeted Mutagenesis in Rice Using CRISPR-Cas System. *Cell Research* 2013, *23*.
23. Sugano, S.S.; Shirakawa, M.; Takagi, J.; Matsuda, Y.; Shimada, T.; Hara-Nishimura, I.; Kohchi, T. CRISPR/Cas9-Mediated Targeted Mutagenesis in the Liverwort Marchantia Polymorpha L. *Plant and Cell Physiology***2014**, *55*, doi:10.1093/pcp/pcu014.
24. Brooks, C.; Nekrasov, V.; Lipppman, Z.B.; van Eck, J. Efficient Gene Editing in Tomato in the First Generation Using the Clustered Regularly Interspaced Short Palindromic Repeats/CRISPR-Associated9 System. *Plant Physiology***2014**, *166*, doi:10.1104/pp.114.247577.
25. Wang, S.; Zhang, S.; Wang, W.; Xiong, X.; Meng, F.; Cui, X. Efficient Targeted Mutagenesis in Potato by the CRISPR/Cas9 System. *Plant Cell Reports* 2015, *34*.
26. Jacobs, T.B.; LaFayette, P.R.; Schmitz, R.J.; Parrott, W.A. Targeted Genome Modifications in Soybean with CRISPR/Cas9. *BMC Biotechnology***2015**, *15*, doi:10.1186/s12896-015-0131-2.
27. Jia, H.; Nian, W. Targeted Genome Editing of Sweet Orange Using Cas9/SgRNA. *PLoS ONE***2014**, *9*, doi:10.1371/journal.pone.0093806.

28. Tripathi, J.N.; Ntui, V.O.; Ron, M.; Muiruri, S.K.; Britt, A.; Tripathi, L. CRISPR/Cas9 Editing of Endogenous Banana Streak Virus in the B Genome of Musa Spp. Overcomes a Major Challenge in Banana Breeding. *Communications Biology* **2019**, *2*, doi:10.1038/s42003-019-0288-7.
29. Park, S. il; Kim, H. bin; Jeon, H.J.; Kim, H. Agrobacterium-Mediated Capsicum Annuum Gene Editing in Two Cultivars, Hot Pepper CM334 and Bell Pepper Dempsey. *International Journal of Molecular Sciences* **2021**, *22*, doi:10.3390/ijms22083921.
30. Oz, M.T.; Altpeter, A.; Karan, R.; Merotto, A.; Altpeter, F. CRISPR/Cas9-Mediated Multi-Allelic Gene Targeting in Sugarcane Confers Herbicide Tolerance. *Frontiers in Genome Editing* **2021**, *3*, doi:10.3389/fgeed.2021.673566.
31. Wang, W.; Pan, Q.; He, F.; Akhunova, A.; Chao, S.; Trick, H.; Akhunov, E. Transgenerational CRISPR-Cas9 Activity Facilitates Multiplex Gene Editing in Allopolyploid Wheat. *The CRISPR Journal* **2018**, *1*, doi:10.1089/crispr.2017.0010.
32. Gao, W.; Long, L.; Tian, X.; Xu, F.; Liu, J.; Singh, P.K.; Botella, J.R.; Song, C. Genome Editing in Cotton with the CRISPR/Cas9 System. *Frontiers in Plant Science* **2017**, *8*, doi:10.3389/fpls.2017.01364.
33. Char, S.N.; Neelakandan, A.K.; Nahampun, H.; Frame, B.; Main, M.; Spalding, M.H.; Becraft, P.W.; Meyers, B.C.; Walbot, V.; Wang, K.; et al. An Agrobacterium-Delivered CRISPR/Cas9 System for High-Frequency Targeted Mutagenesis in Maize. *Plant Biotechnology Journal* **2017**, *15*, doi:10.1111/pbi.12611.
34. Zafar, S.A.; Zaidi, S.S.E.A.; Gaba, Y.; Singla-Pareek, S.L.; Dhankher, O.P.; Li, X.; Mansoor, S.; Pareek, A. Engineering Abiotic Stress Tolerance via CRISPR/Cas-Mediated Genome Editing. *Journal of Experimental Botany* 2020, *71*.
35. Mushtaq, M.; Bhat, J.A.; Mir, Z.A.; Sakina, A.; Ali, S.; Singh, A.K.; Tyagi, A.; Salgotra, R.K.; Dar, A.A.; Bhat, R. CRISPR/Cas Approach: A New Way of Looking at Plant-Abiotic Interactions. *Journal of Plant Physiology* 2018, *224–225*.
36. Lou, D.; Wang, H.; Liang, G.; Yu, D. OsSAPK2 Confers Abscisic Acid Sensitivity and Tolerance to Drought Stress in Rice. *Frontiers in Plant Science* **2017**, *8*, doi:10.3389/fpls.2017.00993.
37. Debbarma, J.; Sarki, Y.N.; Saikia, B.; Boruah, H.P.D.; Singha, D.L.; Chikkaputtaiah, C. Ethylene Response Factor (ERF) Family Proteins in Abiotic Stresses and CRISPR–Cas9 Genome Editing of ERFs for Multiple Abiotic Stress Tolerance in Crop Plants: A Review. *Molecular Biotechnology* 2019, *61*.
38. Shi, J.; Gao, H.; Wang, H.; Lafitte, H.R.; Archibald, R.L.; Yang, M.; Hakimi, S.M.; Mo, H.; Habben, J.E. ARGOS8 Variants Generated by CRISPR-Cas9 Improve Maize Grain Yield under Field Drought Stress Conditions. *Plant Biotechnology Journal* **2017**, *15*, doi:10.1111/pbi.12603.
39. Li, R.; Liu, C.; Zhao, R.; Wang, L.; Chen, L.; Yu, W.; Zhang, S.; Sheng, J.; Shen, L. CRISPR/Cas9-Mediated SlNPR1 Mutagenesis Reduces Tomato Plant Drought Tolerance. *BMC Plant Biology* **2019**, *19*, doi:10.1186/s12870-018-1627-4.
40. Marraffini, L.A.; Sontheimer, E.J. Self versus Non-Self Discrimination during CRISPR RNA-Directed Immunity. *Nature* **2010**, *463*, doi:10.1038/nature08703.
41. Cong, L.; Ran, F.A.; Cox, D.; Lin, S.; Barretto, R.; Habib, N.; Hsu, P.D.; Wu, X.; Jiang, W.; Marraffini, L.A.; et al. Multiplex Genome Engineering Using CRISPR/Cas Systems. *Science (1979)* **2013**, *339*, doi:10.1126/science.1231143.
42. Mei, Y.; Wang, Y.; Chen, H.; Sun, Z.S.; Ju, X. da Recent Progress in CRISPR/Cas9 Technology. *Journal of Genetics and Genomics* 2016, *43*.
43. Liu, H.; Hu, M.; Wang, Q.; Cheng, L.; Zhang, Z. Role of Papain-like Cysteine Proteases in Plant Development. *Frontiers in Plant Science* 2018, *871*.
44. Yang, H.; Ren, S.; Yu, S.; Pan, H.; Li, T.; Ge, S.; Zhang, J.; Xia, N. Methods Favoring Homology-Directed Repair Choice in Response to Crispr/Cas9 Induced-Double Strand Breaks. *International Journal of Molecular Sciences* 2020, *21*.
45. Puchta, H. The Repair of Double-Strand Breaks in Plants: Mechanisms and Consequences for Genome Evolution. *Journal of Experimental Botany* 2005, *56*.
46. Li, J.; Zhang, H.; Si, X.; Tian, Y.; Chen, K.; Liu, J.; Chen, H.; Gao, C. Generation of Thermosensitive Male-Sterile Maize by Targeted Knockout of the ZmTMS5 Gene. *Journal of Genetics and Genomics* 2017, *44*.

47. Veillet, F.; Perrot, L.; Chauvin, L.; Kermarrec, M.P.; Guyon-Debast, A.; Chauvin, J.E.; Nogué, F.; Mazier, M. Transgene-Free Genome Editing in Tomato and Potato Plants Using Agrobacterium-Mediated Delivery of a CRISPR/Cas9 Cytidine Base Editor. *International Journal of Molecular Sciences***2019**, *20*, doi:10.3390/ijms20020402.
48. Yin, K.; Gao, C.; Qiu, J.L. Progress and Prospects in Plant Genome Editing. *Nature Plants* 2017, *3*.
49. Davies, K. All about That Base Editing. *Genetic Engineering and Biotechnology News***2019**, *39*, doi:10.1089/gen.39.05.16.
50. Komor, A.C.; Kim, Y.B.; Packer, M.S.; Zuris, J.A.; Liu, D.R. Programmable Editing of a Target Base in Genomic DNA without Double-Stranded DNA Cleavage. *Nature***2016**, *533*, doi:10.1038/nature17946.
51. Yu, Q.; Powles, S.B. Resistance to AHAS Inhibitor Herbicides: Current Understanding. *Pest Management Science* 2014, *70*.
52. Shimatani, Z.; Kashojiya, S.; Takayama, M.; Terada, R.; Arazoe, T.; Ishii, H.; Teramura, H.; Yamamoto, T.; Komatsu, H.; Miura, K.; et al. Targeted Base Editing in Rice and Tomato Using a CRISPR-Cas9 Cytidine Deaminase Fusion. *Nature Biotechnology***2017**, *35*, doi:10.1038/nbt.3833.
53. Chen, H.J.; Su, C.T.; Lin, C.H.; Huang, G.J.; Lin, Y.H. Expression of Sweet Potato Cysteine Protease SPCP2 Altered Developmental Characteristics and Stress Responses in Transgenic Arabidopsis Plants. *Journal of Plant Physiology***2010**, *167*, doi:10.1016/j.jplph.2010.01.005.
54. Anzalone, A. v.; Randolph, P.B.; Davis, J.R.; Sousa, A.A.; Koblan, L.W.; Levy, J.M.; Chen, P.J.; Wilson, C.; Newby, G.A.; Raguram, A.; et al. Search-and-Replace Genome Editing without Double-Strand Breaks or Donor DNA. *Nature***2019**, *576*, doi:10.1038/s41586-019-1711-4.
55. Jiang, Y.Y.; Chai, Y.P.; Lu, M.H.; Han, X.L.; Lin, Q.; Zhang, Y.; Zhang, Q.; Zhou, Y.; Wang, X.C.; Gao, C.; et al. Prime Editing Efficiently Generates W542L and S621I Double Mutations in Two ALS Genes in Maize. *Genome Biology***2020**, *21*, doi:10.1186/s13059-020-02170-5.
56. Lin, Q.; Zong, Y.; Xue, C.; Wang, S.; Jin, S.; Zhu, Z.; Wang, Y.; Anzalone, A. v.; Raguram, A.; Doman, J.L.; et al. Prime Genome Editing in Rice and Wheat. *Nature Biotechnology***2020**, *38*, doi:10.1038/s41587-020-0455-x.
57. Lu, Y.; Tian, Y.; Shen, R.; Yao, Q.; Zhong, D.; Zhang, X.; Zhu, J.K. Precise Genome Modification in Tomato Using an Improved Prime Editing System. *Plant Biotechnology Journal***2021**, *19*, doi:10.1111/pbi.13497.
58. Gu, X.; Liu, L.; Zhang, H. Transgene-Free Genome Editing in Plants. *Frontiers in Genome Editing***2021**, *3*, doi:10.3389/fgeed.2021.805317.
59. Kim, S.; Kim, D.; Cho, S.W.; Kim, J.; Kim, J.S. Highly Efficient RNA-Guided Genome Editing in Human Cells via Delivery of Purified Cas9 Ribonucleoproteins. *Genome Research***2014**, *24*, doi:10.1101/gr.171322.113.
60. Mao, Y.; Botella, J.R.; Liu, Y.; Zhu, J.K. Gene Editing in Plants: Progress and Challenges. *National Science Review* 2019, *6*.
61. Wolter, F.; Puchta, H. Knocking out Consumer Concerns and Regulator's Rules: Efficient Use of CRISPR/Cas Ribonucleoprotein Complexes for Genome Editing in Cereals. *Genome Biology* 2017, *18*.
62. Tsanova, T.; Stefanova, L.; Topalova, L.; Atanasov, A.; Pantchev, I. DNA-Free Gene Editing in Plants: A Brief Overview. *Biotechnology and Biotechnological Equipment* 2021, *35*.
63. Woo, J.W.; Kim, J.; Kwon, S. il; Corvalán, C.; Cho, S.W.; Kim, H.; Kim, S.G.; Kim, S.T.; Choe, S.; Kim, J.S. DNA-Free Genome Editing in Plants with Preassembled CRISPR-Cas9 Ribonucleoproteins. *Nature Biotechnology***2015**, *33*, doi:10.1038/nbt.3389.
64. Zhang, Y.; Liang, Z.; Zong, Y.; Wang, Y.; Liu, J.; Chen, K.; Qiu, J.L.; Gao, C. Efficient and Transgene-Free Genome Editing in Wheat through Transient Expression of CRISPR/Cas9 DNA or RNA. *Nature Communications***2016**, *7*, doi:10.1038/ncomms12617.
65. Rather, G.A.; Ayzenshtat, D.; Teper-Bamnolker, P.; Kumar, M.; Forotan, Z.; Eshel, D.; Bocobza, S. Advances in Protoplast Transfection Promote Efficient CRISPR/Cas9-Mediated Genome Editing in Tetraploid Potato. *Planta***2022**, *256*, doi:10.1007/s00425-022-03933-z.
66. McCarty, N.S.; Graham, A.E.; Studená, L.; Ledesma-Amaro, R. Multiplexed CRISPR Technologies for Gene Editing and Transcriptional Regulation. *Nature Communications* 2020, *11*.

67. Wang, C.; Liu, Q.; Shen, Y.; Hua, Y.; Wang, J.; Lin, J.; Wu, M.; Sun, T.; Cheng, Z.; Mercier, R.; et al. Clonal Seeds from Hybrid Rice by Simultaneous Genome Engineering of Meiosis and Fertilization Genes. *Nature Biotechnology* **2019**, *37*, doi:10.1038/s41587-018-0003-0.
68. Minkenberg, B.; Wheatley, M.; Yang, Y. CRISPR/Cas9-Enabled Multiplex Genome Editing and Its Application. In *Progress in Molecular Biology and Translational Science*; 2017; Vol. 149.
69. Armario Najera, V.; Twyman, R.M.; Christou, P.; Zhu, C. Applications of Multiplex Genome Editing in Higher Plants. *Current Opinion in Biotechnology* 2019, *59*.
70. Lu, H.P.; Liu, S.M.; Xu, S.L.; Chen, W.Y.; Zhou, X.; Tan, Y.Y.; Huang, J.Z.; Shu, Q.Y. CRISPR-S: An Active Interference Element for a Rapid and Inexpensive Selection of Genome-Edited, Transgene-Free Rice Plants. *Plant Biotechnology Journal* 2017, *15*.
71. Jacobs, T.B.; Zhang, N.; Patel, D.; Martin, G.B. Generation of a Collection of Mutant Tomato Lines Using Pooled CRISPR Libraries. *Plant Physiology* **2017**, *174*, doi:10.1104/pp.17.00489.
72. Meng, X.; Yu, H.; Zhang, Y.; Zhuang, F.; Song, X.; Gao, S.; Gao, C.; Li, J. Construction of a Genome-Wide Mutant Library in Rice Using CRISPR/Cas9. *Molecular Plant* 2017, *10*.
73. Ricroch, A.; Clairand, P.; Harwood, W. Use of CRISPR Systems in Plant Genome Editing: Toward New Opportunities in Agriculture. *Emerging Topics in Life Sciences* 2017, *1*.
74. Tian, H.; Chen, S.; Yang, W.; Wang, T.; Zheng, K.; Wang, Y.; Cheng, Y.; Zhang, N.; Liu, S.; Li, D.; et al. A Novel Family of Transcription Factors Conserved in Angiosperms Is Required for ABA Signalling. *Plant Cell and Environment* **2017**, *40*, doi:10.1111/pce.13058.
75. Chen, S.; Zhang, N.; Zhou, G.; Hussain, S.; Ahmed, S.; Tian, H.; Wang, S. Knockout of the Entire Family of AITR Genes in Arabidopsis Leads to Enhanced Drought and Salinity Tolerance without Fitness Costs. *BMC Plant Biology* **2021**, *21*, doi:10.1186/s12870-021-02907-9.
76. Bhattacharya, A.; Parkhi, V.; Char, B. Genome Editing for Crop Improvement: A Perspective from India. *In Vitro Cellular and Developmental Biology - Plant* 2021, *57*.
77. Santosh Kumar, V. v.; Verma, R.K.; Yadav, S.K.; Yadav, P.; Watts, A.; Rao, M. v.; Chinnusamy, V. CRISPR-Cas9 Mediated Genome Editing of Drought and Salt Tolerance (OsDST) Gene in Indica Mega Rice Cultivar MTU1010. *Physiology and Molecular Biology of Plants* **2020**, *26*, doi:10.1007/s12298-020-00819-w.
78. Yue, E.; Cao, H.; Liu, B. Osmir535, a Potential Genetic Editing Target for Drought and Salinity Stress Tolerance in Oryza Sativa. *Plants* 2020, *9*.
79. Osakabe, Y.; Watanabe, T.; Sugano, S.S.; Ueta, R.; Ishihara, R.; Shinozaki, K.; Osakabe, K. Optimization of CRISPR/Cas9 Genome Editing to Modify Abiotic Stress Responses in Plants. *Scientific Reports* **2016**, *6*, doi:10.1038/srep26685.
80. Roca Paixão, J.F.; Gillet, F.X.; Ribeiro, T.P.; Bournaud, C.; Lourenço-Tessutti, I.T.; Noriega, D.D.; Melo, B.P. de; de Almeida-Engler, J.; Grossi-de-Sa, M.F. Improved Drought Stress Tolerance in Arabidopsis by CRISPR/DCas9 Fusion with a Histone AcetylTransferase. *Scientific Reports* **2019**, *9*, doi:10.1038/s41598-019-44571-y.
81. Park, J.J.; Dempewolf, E.; Zhang, W.; Wang, Z.Y. RNA-Guided Transcriptional Activation via CRISPR/DCas9 Mimics Overexpression Phenotypes in Arabidopsis. *PLoS ONE* **2017**, *12*, doi:10.1371/journal.pone.0179410.
82. Zhao, Y.; Zhang, C.; Liu, W.; Gao, W.; Liu, C.; Song, G.; Li, W.X.; Mao, L.; Chen, B.; Xu, Y.; et al. An Alternative Strategy for Targeted Gene Replacement in Plants Using a Dual-SgRNA/Cas9 Design. *Scientific Reports* **2016**, *6*, doi:10.1038/srep23890.
83. Wu, J.; Yan, G.; Duan, Z.; Wang, Z.; Kang, C.; Guo, L.; Liu, K.; Tu, J.; Shen, J.; Yi, B.; et al. Roles of the Brassica Napus DELLA Protein BnaA6.RGA, in Modulating Drought Tolerance by Interacting With the ABA Signaling Component BnaA10.ABF2. *Frontiers in Plant Science* **2020**, *11*, doi:10.3389/fpls.2020.00577.
84. Badhan, S.; Ball, A.S.; Mantri, N. First Report of CRISPR/Cas9 Mediated DNA-Free Editing of 4CL and RVE7 Genes in Chickpea Protoplasts. *International Journal of Molecular Sciences* **2021**, *22*, doi:10.3390/ijms22010396.

85. Du, Y.T.; Zhao, M.J.; Wang, C.T.; Gao, Y.; Wang, Y.X.; Liu, Y.W.; Chen, M.; Chen, J.; Zhou, Y. bin; Xu, Z.S.; et al. Identification and Characterization of GmMYB118 Responses to Drought and Salt Stress. *BMC Plant Biology* **2018**, *18*, doi:10.1186/s12870-018-1551-7.
86. Ogata, T.; Ishizaki, T.; Fujita, M.; Fujita, Y. CRISPR/Cas9-Targeted Mutagenesis of OsERA1 Confers Enhanced Responses to Abscisic Acid and Drought Stress and Increased Primary Root Growth under Nonstressed Conditions in Rice. *PLoS ONE* **2020**, *15*, doi:10.1371/journal.pone.0243376.
87. Liao, S.; Qin, X.; Luo, L.; Han, Y.; Wang, X.; Usman, B.; Nawaz, G.; Zhao, N.; Liu, Y.; Li, R. CRISPR/Cas9-Induced Mutagenesis of Semi-Rolled Leaf1,2 Confers Curled Leaf Phenotype and Drought Tolerance by Influencing Protein Expression Patterns and ROS Scavenging in Rice (Oryza Sativa L.). *Agronomy* **2019**, *9*, doi:10.3390/agronomy9110728.
88. Shim, J.S.; Oh, N.; Chung, P.J.; Kim, Y.S.; Choi, Y. do; Kim, J.K. Overexpression of OsNAC14 Improves Drought Tolerance in Rice. *Frontiers in Plant Science* **2018**, *9*, doi:10.3389/fpls.2018.00310.
89. Qin, Q.; Wang, Y.; Huang, L.; Du, F.; Zhao, X.; Li, Z.; Wang, W.; Fu, B. A U-Box E3 Ubiquitin Ligase OsPUB67 Is Positively Involved in Drought Tolerance in Rice. *Plant Molecular Biology* **2020**, *102*, 89–107, doi:10.1007/s11103-019-00933-8.
90. Liu, L.; Zhang, J.; Xu, J.; Li, Y.; Guo, L.; Wang, Z.; Zhang, X.; Zhao, B.; Guo, Y.D.; Zhang, N. CRISPR/Cas9 Targeted Mutagenesis of SlLBD40, a Lateral Organ Boundaries Domain Transcription Factor, Enhances Drought Tolerance in Tomato. *Plant Science* **2020**, *301*, doi:10.1016/j.plantsci.2020.110683.
91. Kim, D.; Alptekin, B.; Budak, H. CRISPR/Cas9 Genome Editing in Wheat. *Functional and Integrative Genomics* **2018**, *18*, doi:10.1007/s10142-017-0572-x.
92. Li, P.; Li, X.; Jiang, M. CRISPR/Cas9-Mediated Mutagenesis of WRKY3 and WRKY4 Function Decreases Salt and Me-JA Stress Tolerance in Arabidopsis Thaliana. *Molecular Biology Reports* **2021**, *48*, doi:10.1007/s11033-021-06541-4.
93. Kim, S.T.; Choi, M.; Bae, S.J.; Kim, J.S. The Functional Association of Acqos/Victr with Salt Stress Resistance in Arabidopsis Thaliana Was Confirmed by Crispr-Mediated Mutagenesis. *International Journal of Molecular Sciences* **2021**, *22*, doi:10.3390/ijms222111389.
94. Curtin, S.J.; Xiong, Y.; Michno, J.M.; Campbell, B.W.; Stec, A.O.; Čermák, T.; Starker, C.; Voytas, D.F.; Eamens, A.L.; Stupar, R.M. CRISPR/Cas9 and TALENs Generate Heritable Mutations for Genes Involved in Small RNA Processing of Glycine Max and Medicago Truncatula. *Plant Biotechnology Journal* **2018**, *16*, doi:10.1111/pbi.12857.
95. Wang, T.; Xun, H.; Wang, W.; Ding, X.; Tian, H.; Hussain, S.; Dong, Q.; Li, Y.; Cheng, Y.; Wang, C.; et al. Mutation of GmAITR Genes by CRISPR/Cas9 Genome Editing Results in Enhanced Salinity Stress Tolerance in Soybean. *Frontiers in Plant Science* **2021**, *12*, doi:10.3389/fpls.2021.779598.
96. Duan, Y.B.; Li, J.; Qin, R.Y.; Xu, R.F.; Li, H.; Yang, Y.C.; Ma, H.; Li, L.; Wei, P.C.; Yang, J.B. Identification of a Regulatory Element Responsible for Salt Induction of Rice OsRAV2 through Ex Situ and in Situ Promoter Analysis. *Plant Molecular Biology* **2016**, *90*, doi:10.1007/s11103-015-0393-z.
97. Zhang, A.; Liu, Y.; Wang, F.; Li, T.; Chen, Z.; Kong, D.; Bi, J.; Zhang, F.; Luo, X.; Wang, J.; et al. Enhanced Rice Salinity Tolerance via CRISPR/Cas9-Targeted Mutagenesis of the OsRR22 Gene. *Molecular Breeding* **2019**, *39*, doi:10.1007/s11032-019-0954-y.
98. Zhang, X.; Long, Y.; Huang, J.; Xia, J. OsNAC45 Is Involved in ABA Response and Salt Tolerance in Rice. *Rice* **2020**, *13*, doi:10.1186/s12284-020-00440-1.
99. Zeng, D.D.; Yang, C.C.; Qin, R.; Alamin, M.; Yue, E.K.; Jin, X.L.; Shi, C.H. A Guanine Insert in OsBBS1 Leads to Early Leaf Senescence and Salt Stress Sensitivity in Rice (Oryza Sativa L.). *Plant Cell Reports* **2018**, *37*, doi:10.1007/s00299-018-2280-y.
100. Yin, W.; Xiao, Y.; Niu, M.; Meng, W.; Li, L.; Zhang, X.; Liu, D.; Zhang, G.; Qian, Y.; Sun, Z.; et al. ARGONAUTE2 Enhances Grain Length and Salt Tolerance by Activating BIG GRAIN3 to Modulate Cytokinin Distribution in Rice. *Plant Cell* **2020**, *32*, doi:10.1105/tpc.19.00542.

101. Bo, W.; Zhaohui, Z.; Huanhuan, Z.; Xia, W.; Binglin, L.; Lijia, Y.; Xiangyan, H.; Deshui, Y.; Xuelian, Z.; Chunguo, W.; et al. Targeted Mutagenesis of NAC Transcription Factor Gene, OsNAC041, Leading to Salt Sensitivity in Rice. *Rice Science* **2019**, *26*, doi:10.1016/j.rsci.2018.12.005.
102. Alamin, A.; Ullah Jan, S.; Xia, J.; Xiang, C.-B. Loss of Rice PARAQUAT TOLERANCE 3 Confers Enhanced Resistance to Abiotic Stresses and Increases Grain Yield in Field Characterization of Arabidopsis Paraquat Tolerant Mutants View Project., doi:10.1101/2020.02.22.961151.
103. Mo, W.; Tang, W.; Du, Y.; Jing, Y.; Bu, Q.; Lin, R. PHYTOCHROME-INTERACTING FACTOR-LIKE14 and SLENDER RICE1 Interaction Controls Seedling Growth under Salt Stress1[OPEN]. *Plant Physiology* **2020**, *184*, doi:10.1104/PP.20.00024.
104. Lan, T.; Zheng, Y.; Su, Z.; Yu, S.; Song, H.; Zheng, X.; Lin, G.; Wu, W. OsSPL10, a SBP-Box Gene, Plays a Dual Role in Salt Tolerance and Trichome Formation in Rice (Oryza Sativa L.). *G3: Genes, Genomes, Genetics* **2019**, *9*, doi:10.1534/g3.119.400700.
105. Qin, H.; Wang, J.; Chen, X.; Wang, F.; Peng, P.; Zhou, Y.; Miao, Y.; Zhang, Y.; Gao, Y.; Qi, Y.; et al. Rice OsDOF15 Contributes to Ethylene-Inhibited Primary Root Elongation under Salt Stress. *New Phytologist* **2019**, *223*, doi:10.1111/nph.15824.
106. Chen, G.; Hu, J.; Dong, L.; Zeng, D.; Guo, L.; Zhang, G.; Zhu, L.; Qian, Q. The Tolerance of Salinity in Rice Requires the Presence of a Functional Copy of FLN2. *Biomolecules* **2020**, *10*, doi:10.3390/biom10010017.
107. Makhotenko, A. v.; Khromov, A. v.; Snigir, E.A.; Makarova, S.S.; Makarov, V. v.; Suprunova, T.P.; Kalinina, N.O.; Taliansky, M.E. Functional Analysis of Coilin in Virus Resistance and Stress Tolerance of Potato Solanum Tuberosum Using CRISPR-Cas9 Editing. *Doklady Biochemistry and Biophysics* **2019**, *484*, doi:10.1134/S1607672919010241.
108. Tran, M.T.; Doan, D.T.H.; Kim, J.; Song, Y.J.; Sung, Y.W.; Das, S.; Kim, E.J.; Son, G.H.; Kim, S.H.; van Vu, T.; et al. CRISPR/Cas9-Based Precise Excision of SlHyPRP1 Domain(s) to Obtain Salt Stress-Tolerant Tomato. *Plant Cell Reports* **2021**, *40*, doi:10.1007/s00299-020-02622-z.
109. Zheng, M.; Lin, J.; Liu, X.; Chu, W.; Li, J.; Gao, Y.; An, K.; Song, W.; Xin, M.; Yao, Y.; et al. Histone Acetyltransferase TaHAG1 Acts as a Crucial Regulator to Strengthen Salt Tolerance of Hexaploid Wheat. *Plant Physiology* **2021**, *186*, doi:10.1093/plphys/kiab187.
110. Fang, Y.; Xiong, L. General Mechanisms of Drought Response and Their Application in Drought Resistance Improvement in Plants. *Cellular and Molecular Life Sciences* 2015, *72*.
111. Kumar, M.; Yusuf, M.A.; Yadav, P.; Narayan, S.; Kumar, M. Overexpression of Chickpea Defensin Gene Confers Tolerance to Water-Deficit Stress in Arabidopsis Thaliana. *Frontiers in Plant Science* **2019**, *10*, doi:10.3389/fpls.2019.00290.
112. Singh, D.; Laxmi, A. Transcriptional Regulation of Drought Response: A Tortuous Network of Transcriptional Factors. *Frontiers in Plant Science* 2015, *6*.
113. Wang, L.; Chen, L.; Li, R.; Zhao, R.; Yang, M.; Sheng, J.; Shen, L. Reduced Drought Tolerance by CRISPR/Cas9-Mediated SlMAPK3 Mutagenesis in Tomato Plants. *Journal of Agricultural and Food Chemistry* **2017**, *65*, doi:10.1021/acs.jafc.7b02745.
114. Hirai, M.Y.; Sugiyama, K.; Sawada, Y.; Tohge, T.; Obayashi, T.; Suzuki, A.; Araki, R.; Sakurai, N.; Suzuki, H.; Aoki, K.; et al. Omics-Based Identification of Arabidopsis Myb Transcription Factors Regulating Aliphatic Glucosinolate Biosynthesis. *Proc Natl Acad Sci U S A* **2007**, *104*, doi:10.1073/pnas.0611629104.
115. Prusty, M.R.; Kim, S.R.; Vinarao, R.; Entila, F.; Egdane, J.; Diaz, M.G.Q.; Jena, K.K. Newly Identified Wild Rice Accessions Conferring High Salt Tolerance Might Use a Tissue Tolerance Mechanism in Leaf. *Frontiers in Plant Science* **2018**, *9*, doi:10.3389/fpls.2018.00417.
116. Julkowska, M.M.; Testerink, C. Tuning Plant Signaling and Growth to Survive Salt. *Trends in Plant Science* 2015, *20*.
117. Tubiello, F.N.; Soussana, J.F.; Howden, S.M. Crop and Pasture Response to Climate Change. *Proc Natl Acad Sci U S A* 2007, *104*.
118. Awasthi, R.; Bhandari, K.; Nayyar, H. Temperature Stress and Redox Homeostasis in Agricultural Crops. *Frontiers in Environmental Science* 2015, *3*.

119. Parmar, N.; Singh, K.H.; Sharma, D.; Singh, L.; Kumar, P.; Nanjundan, J.; Khan, Y.J.; Chauhan, D.K.; Thakur, A.K. Genetic Engineering Strategies for Biotic and Abiotic Stress Tolerance and Quality Enhancement in Horticultural Crops: A Comprehensive Review. *3 Biotech* 2017, *7*.
120. Pan, C.; Ye, L.; Qin, L.; Liu, X.; He, Y.; Wang, J.; Chen, L.; Lu, G. CRISPR/Cas9-Mediated Efficient and Heritable Targeted Mutagenesis in Tomato Plants in the First and Later Generations. *Scientific Reports* **2016**, *6*, doi:10.1038/srep24765.
121. Klap, C.; Yeshayahou, E.; Bolger, A.M.; Arazi, T.; Gupta, S.K.; Shabtai, S.; Usadel, B.; Salts, Y.; Barg, R. Tomato Facultative Parthenocarpy Results from SlAGAMOUS-LIKE 6 Loss of Function. *Plant Biotechnology Journal* **2017**, *15*, doi:10.1111/pbi.12662.
122. Yu, W.; Wang, L.; Zhao, R.; Sheng, J.; Zhang, S.; Li, R.; Shen, L. Knockout of SlMAPK3 Enhances Tolerance to Heat Stress Involving ROS Homeostasis in Tomato Plants. *BMC Plant Biology* **2019**, *19*, doi:10.1186/s12870-019-1939-z.
123. Yin, Y.; Qin, K.; Song, X.; Zhang, Q.; Zhou, Y.; Xia, X.; Yu, J. BZR1 Transcription Factor Regulates Heat Stress Tolerance through FERONIA Receptor-like Kinase-Mediated Reactive Oxygen Species Signaling in Tomato. *Plant and Cell Physiology* **2018**, *59*, doi:10.1093/pcp/pcy146.
124. Qiu, Z.; Kang, S.; He, L.; Zhao, J.; Zhang, S.; Hu, J.; Zeng, D.; Zhang, G.; Dong, G.; Gao, Z.; et al. The Newly Identified Heat-Stress Sensitive Albino 1 Gene Affects Chloroplast Development in Rice. *Plant Science* **2018**, *267*, doi:10.1016/j.plantsci.2017.11.015.
125. Bertier, L.D.; Ron, M.; Huo, H.; Bradford, K.J.; Britt, A.B.; Michelmore, R.W. High-Resolution Analysis of the Efficiency, Heritability, and Editing Outcomes of CRISPR/Cas9-Induced Modifications of NCED4 in Lettuce (Lactuca Sativa). *G3: Genes, Genomes, Genetics* **2018**, *8*, 1513–1521, doi:10.1534/g3.117.300396.
126. Li, B.; Liang, S.; Alariqi, M.; Wang, F.; Wang, G.; Wang, Q.; Xu, Z.; Yu, L.; Naeem Zafar, M.; Sun, L.; et al. The Application of Temperature Sensitivity CRISPR/LbCpf1 (LbCas12a) Mediated Genome Editing in Allotetraploid Cotton (G. Hirsutum) and Creation of Nontransgenic, Gossypol-Free Cotton. *Plant Biotechnology Journal* **2021**, *19*, doi:10.1111/pbi.13470.
127. Nandy, S.; Pathak, B.; Zhao, S.; Srivastava, V. Heat-Shock-Inducible CRISPR/Cas9 System Generates Heritable Mutations in Rice. *Plant Direct* **2019**, *3*, doi:10.1002/pld3.145.
128. Wang, B.; Zhong, Z.; Wang, X.; Han, X.; Yu, D.; Wang, C.; Song, W.; Zheng, X.; Chen, C.; Zhang, Y. Knockout of the OsNAC006 Transcription Factor Causes Drought and Heat Sensitivity in Rice. *International Journal of Molecular Sciences* **2020**, *21*, doi:10.3390/ijms21072288.
129. Miao, C.; Xiao, L.; Hua, K.; Zou, C.; Zhao, Y.; Bressan, R.A.; Zhu, J.K. Mutations in a Subfamily of Abscisic Acid Recepto Genes Promote Rice Growth and Productivity. *Proc Natl Acad Sci U S A* **2018**, *115*, doi:10.1073/pnas.1804774115.
130. Hu, Z.; Li, J.; Ding, S.; Cheng, F.; Li, X.; Jiang, Y.; Yu, J.; Foyer, C.H.; Shi, K. The Protein Kinase CPK28 Phosphorylates Ascorbate Peroxidase and Enhances Thermotolerance in Tomato. *Plant Physiology* **2021**, *186*, doi:10.1093/PLPHYS/KIAB120.
131. Shen, C.; Que, Z.; Xia, Y.; Tang, N.; Li, D.; He, R.; Cao, M. Knock out of the Annexin Gene OsAnn3 via CRISPR/Cas9-Mediated Genome Editing Decreased Cold Tolerance in Rice. *Journal of Plant Biology* **2017**, *60*, doi:10.1007/s12374-016-0400-1.
132. Zeng, Y.; Wen, J.; Zhao, W.; Wang, Q.; Huang, W. Rational Improvement of Rice Yield and Cold Tolerance by Editing the Three Genes OsPIN5b, GS3, and OsMYB30 With the CRISPR–Cas9 System. *Frontiers in Plant Science* **2020**, *10*, doi:10.3389/fpls.2019.01663.
133. Nawaz, G.; Han, Y.; Usman, B.; Liu, F.; Qin, B.; Li, R. Knockout of OsPRP1, a Gene Encoding Proline-Rich Protein, Confers Enhanced Cold Sensitivity in Rice (Oryza Sativa L.) at the Seedling Stage. *3 Biotech* **2019**, *9*, doi:10.1007/s13205-019-1787-4.
134. Li, R.; Zhang, L.; Wang, L.; Chen, L.; Zhao, R.; Sheng, J.; Shen, L. Reduction of Tomato-Plant Chilling Tolerance by CRISPR-Cas9-Mediated SlCBF1 Mutagenesis. *Journal of Agricultural and Food Chemistry* **2018**, *66*, doi:10.1021/acs.jafc.8b02177.
135. Hannah, M.A.; Wiese, D.; Freund, S.; Fiehn, O.; Heyer, A.G.; Hincha, D.K. Natural Genetic Variation of Freezing Tolerance in Arabidopsis. *Plant Physiology* **2006**, *142*, doi:10.1104/pp.106.081141.

136. Koseki, M.; Kitazawa, N.; Yonebayashi, S.; Maehara, Y.; Wang, Z.X.; Minobe, Y. Identification and Fine Mapping of a Major Quantitative Trait Locus Originating from Wild Rice, Controlling Cold Tolerance at the Seedling Stage. *Molecular Genetics and Genomics* **2010**, *284*, doi:10.1007/s00438-010-0548-1.
137. Shakiba, E.; Edwards, J.D.; Jodari, F.; Duke, S.E.; Baldo, A.M.; Korniliev, P.; McCouch, S.R.; Eizenga, G.C. Genetic Architecture of Cold Tolerance in Rice (Oryza Sativa) Determined through High Resolution Genome-Wide Analysis. *PLoS ONE* **2017**, *12*, doi:10.1371/journal.pone.0172133.
138. Lv, Y.; Yang, M.; Hu, D.; Yang, Z.; Ma, S.; Li, X.; Xiong, L. The OsMYb30 Transcription Factor Suppresses Cold Tolerance by Interacting with a JAZ Protein and Suppressing β-Amylase Expression1[OPEN]. *Plant Physiology* **2017**, *173*, doi:10.1104/pp.16.01725.
139. Li, T.; Yang, X.; Yu, Y.; Si, X.; Zhai, X.; Zhang, H.; Dong, W.; Gao, C.; Xu, C. Domestication of Wild Tomato Is Accelerated by Genome Editing. *Nature Biotechnology* **2018**, *36*, doi:10.1038/nbt.4273.
140. Chen, L.; Yang, Y.; Liu, C.; Zheng, Y.; Xu, M.; Wu, N.; Sheng, J.; Shen, L. Characterization of WRKY Transcription Factors in Solanum Lycopersicum Reveals Collinearity and Their Expression Patterns under Cold Treatment. *Biochemical and Biophysical Research Communications* **2015**, *464*, doi:10.1016/j.bbrc.2015.07.085.
141. Zhang, Y.; Yu, H.; Yang, X.; Li, Q.; Ling, J.; Wang, H.; Gu, X.; Huang, S.; Jiang, W. CsWRKY46, a WRKY Transcription Factor from Cucumber, Confers Cold Resistance in Transgenic-Plant by Regulating a Set of Cold-Stress Responsive Genes in an ABA-Dependent Manner. *Plant Physiology and Biochemistry* **2016**, *108*, doi:10.1016/j.plaphy.2016.08.013.
142. Ke, L.; Lei, W.; Yang, W.; Wang, J.; Gao, J.; Cheng, J.; Sun, Y.; Fan, Z.; Yu, D. Genome-Wide Identification of Cold Responsive Transcription Factors in Brassica Napus L. *BMC Plant Biology* **2020**, *20*, doi:10.1186/s12870-020-2253-5.
143. Yadav, S.K. Heavy Metals Toxicity in Plants: An Overview on the Role of Glutathione and Phytochelatins in Heavy Metal Stress Tolerance of Plants. *South African Journal of Botany* 2010, *76*.
144. Sruthi, P.; Shackira, A.M.; Puthur, J.T. Heavy Metal Detoxification Mechanisms in Halophytes: An Overview. *Wetlands Ecology and Management* 2017, *25*.
145. Hasanuzzaman, M.; Hakeem, K.R.; Nahar, K.; Alharby, H.F. *Plant Abiotic Stress Tolerance: Agronomic, Molecular and Biotechnological Approaches*; 2019;
146. Baeg, G.J.; Kim, S.H.; Choi, D.M.; Tripathi, S.; Han, Y.J.; Kim, J. il CRISPR/Cas9-Mediated Mutation of 5-Oxoprolinase Gene Confers Resistance to Sulfonamide Compounds in Arabidopsis. *Plant Biotechnology Reports* **2021**, *15*, doi:10.1007/s11816-021-00718-w.
147. Chen, J.; Zou, W.; Meng, L.; Fan, X.; Xu, G.; Ye, G. Advances in the Uptake and Transport Mechanisms and QTLs Mapping of Cadmium in Rice. *International Journal of Molecular Sciences* **2019**, *20*, doi:10.3390/ijms20143417.
148. Tang, L.; Mao, B.; Li, Y.; Lv, Q.; Zhang, L.; Chen, C.; He, H.; Wang, W.; Zeng, X.; Shao, Y.; et al. Knockout of OsNramp5 Using the CRISPR/Cas9 System Produces Low Cd-Accumulating Indica Rice without Compromising Yield. *Scientific Reports* **2017**, *7*, doi:10.1038/s41598-017-14832-9.
149. Nieves-Cordones, M.; Mohamed, S.; Tanoi, K.; Kobayashi, N.I.; Takagi, K.; Vernet, A.; Guiderdoni, E.; Périn, C.; Sentenac, H.; Véry, A.A. Production of Low-Cs+ Rice Plants by Inactivation of the K+ Transporter OsHAK1 with the CRISPR-Cas System. *Plant Journal* **2017**, *92*, doi:10.1111/tpj.13632.
150. Wang, F.Z.; Chen, M.X.; Yu, L.J.; Xie, L.J.; Yuan, L.B.; Qi, H.; Xiao, M.; Guo, W.; Chen, Z.; Yi, K.; et al. OsARM1, an R2R3 MYB Transcription Factor, Is Involved in Regulation of the Response to Arsenic Stress in Rice. *Frontiers in Plant Science* **2017**, *8*, doi:10.3389/fpls.2017.01868.
151. Tang, X.; Lowder, L.G.; Zhang, T.; Malzahn, A.A.; Zheng, X.; Voytas, D.F.; Zhong, Z.; Chen, Y.; Ren, Q.; Li, Q.; et al. A CRISPR-Cpf1 System for Efficient Genome Editing and Transcriptional Repression in Plants. *Nature Plants* **2017**, *3*, doi:10.1038/nplants.2017.18.

Preprints (www.preprints.org) | NOT PEER-REVIEWED | Posted: 8 September 2022 doi:10.20944/preprints202209.0117.v1

152. Zhang, Y.; Iaffaldano, B.; Qi, Y. CRISPR Ribonucleoprotein-Mediated Genetic Engineering in Plants. *Plant Communications* 2021, *2*.
153. Hummel, A.W.; Chauhan, R.D.; Cermak, T.; Mutka, A.M.; Vijayaraghavan, A.; Boyher, A.; Starker, C.G.; Bart, R.; Voytas, D.F.; Taylor, N.J. Allele Exchange at the EPSPS Locus Confers Glyphosate Tolerance in Cassava. *Plant Biotechnology Journal* **2018**, *16*, doi:10.1111/pbi.12868.
154. Zhang, R.; Chen, S.; Meng, X.; Chai, Z.; Wang, D.; Yuan, Y.; Chen, K.; Jiang, L.; Li, J.; Gao, C. Generating Broad-Spectrum Tolerance to ALS-Inhibiting Herbicides in Rice by Base Editing. *Science China Life Sciences* **2021**, *64*, doi:10.1007/s11427-020-1800-5.
155. Wang, F.; Xu, Y.; Li, W.; Chen, Z.; Wang, J.; Fan, F.; Tao, Y.; Jiang, Y.; Zhu, Q.H.; Yang, J. Creating a Novel Herbicide-Tolerance OsALS Allele Using CRISPR/Cas9-Mediated Gene Editing. *Crop Journal* **2021**, *9*, doi:10.1016/j.cj.2020.06.001.
156. Butt, H.; Rao, G.S.; Sedeek, K.; Aman, R.; Kamel, R.; Mahfouz, M. Engineering Herbicide Resistance via Prime Editing in Rice. *Plant Biotechnology Journal* **2020**, *18*, doi:10.1111/pbi.13399.
157. Lyu, Y.-S.; Cao, L.-M.; Huang, W.-Q.; Liu, J.-X.; Lu, H.-P. Disruption of Three Polyamine Uptake Transporter Genes in Rice by CRISPR/Cas9 Gene Editing Confers Tolerance to Herbicide Paraquat. *aBIOTECH* **2022**, doi:10.1007/s42994-022-00075-4.
158. Li, J.; Meng, X.; Zong, Y.; Chen, K.; Zhang, H.; Liu, J.; Li, J.; Gao, C. Gene Replacements and Insertions in Rice by Intron Targeting Using CRISPR-Cas9. *Nature Plants* **2016**, *2*, doi:10.1038/nplants.2016.139.
159. Endo, M.; Mikami, M.; Toki, S. Biallelic Gene Targeting in Rice. *Plant Physiology* **2016**, *170*, doi:10.1104/pp.15.01663.
160. Yang, S.H.; Kim, E.; Park, H.; Koo, Y. Selection of the High Efficient SgRNA for CRISPR-Cas9 to Edit Herbicide Related Genes, PDS, ALS, and EPSPS in Tomato. *Applied Biological Chemistry* **2022**, *65*, doi:10.1186/s13765-022-00679-w.
161. Svitashev, S.; Young, J.K.; Schwartz, C.; Gao, H.; Falco, S.C.; Cigan, A.M. Targeted Mutagenesis, Precise Gene Editing, and Site-Specific Gene Insertion in Maize Using Cas9 and Guide RNA. *Plant Physiology* **2015**, *169*, doi:10.1104/pp.15.00793.
162. Sundström, J.F.; Albihn, A.; Boqvist, S.; Ljungvall, K.; Marstorp, H.; Martiin, C.; Nyberg, K.; Vågsholm, I.; Yuen, J.; Magnusson, U. Future Threats to Agricultural Food Production Posed by Environmental Degradation, Climate Change, and Animal and Plant Diseases - a Risk Analysis in Three Economic and Climate Settings. *Food Security* **2014**, *6*, doi:10.1007/s12571-014-0331-y.
163. Green, J.M. Current State of Herbicides in Herbicide-Resistant Crops. *Pest Management Science* 2014, *70*.
164. Gage, K.L.; Krausz, R.F.; Walters, S.A. Emerging Challenges for Weed Management in Herbicide-Resistant Crops. *Agriculture (Switzerland)* 2019, *9*.
165. Toda, E.; Okamoto, T. CRISPR/Cas9-Based Genome Editing Using Rice Zygotes. *Curr Protoc Plant Biol* **2020**, *5*, doi:10.1002/cppb.20111.
166. Dong, H.; Huang, Y.; Wang, K. The Development of Herbicide Resistance Crop Plants Using Crispr/Cas9-Mediated Gene Editing. *Genes (Basel)* 2021, *12*.
167. Kuang, Y.; Li, S.; Ren, B.; Yan, F.; Spetz, C.; Li, X.; Zhou, X.; Zhou, H. Base-Editing-Mediated Artificial Evolution of OsALS1 In Planta to Develop Novel Herbicide-Tolerant Rice Germplasms. *Molecular Plant* **2020**, *13*, doi:10.1016/j.molp.2020.01.010.
168. Mali, P.; Yang, L.; Esvelt, K.M.; Aach, J.; Guell, M.; DiCarlo, J.E.; Norville, J.E.; Church, G.M. RNA-Guided Human Genome Engineering via Cas9. *Science (1979)* **2013**, *339*, doi:10.1126/science.1232033.
169. Georges, F.; Ray, H. Genome Editing of Crops: A Renewed Opportunity for Food Security. *GM Crops and Food* 2017, *8*.
170. Song, G.; Jia, M.; Chen, K.; Kong, X.; Khattak, B.; Xie, C.; Li, A.; Mao, L. CRISPR/Cas9: A Powerful Tool for Crop Genome Editing. *Crop Journal* 2016, *4*.
171. Barrangou, R. Diversity of CRISPR-Cas Immune Systems and Molecular Machines. *Genome Biology* 2015, *16*.
172. Scheben, A.; Wolter, F.; Batley, J.; Puchta, H.; Edwards, D. Towards CRISPR/CAS Crops – Bringing Together Genomics and Genome Editing. *New Phytologist* 2017, *216*.

Preprints (www.preprints.org) | NOT PEER-REVIEWED | Posted: 8 September 2022 doi:10.20944/preprints202209.0117.v1

173. Wang, C.; Liu, Q.; Shen, Y.; Hua, Y.; Wang, J.; Lin, J.; Wu, M.; Sun, T.; Cheng, Z.; Mercier, R.; et al. Clonal Seeds from Hybrid Rice by Simultaneous Genome Engineering of Meiosis and Fertilization Genes. *Nature Biotechnology* **2019**, *37*, doi:10.1038/s41587-018-0003-0.
174. Hilton, I.B.; D'Ippolito, A.M.; Vockley, C.M.; Thakore, P.I.; Crawford, G.E.; Reddy, T.E.; Gersbach, C.A. Epigenome Editing by a CRISPR-Cas9-Based Acetyltransferase Activates Genes from Promoters and Enhancers. *Nature Biotechnology* **2015**, *33*, doi:10.1038/nbt.3199.
175. Konermann, S.; Brigham, M.D.; Trevino, A.E.; Joung, J.; Abudayyeh, O.O.; Barcena, C.; Hsu, P.D.; Habib, N.; Gootenberg, J.S.; Nishimasu, H.; et al. Genome-Scale Transcriptional Activation by an Engineered CRISPR-Cas9 Complex. *Nature* **2015**, *517*, doi:10.1038/nature14136.
176. Larson, M.H.; Gilbert, L.A.; Wang, X.; Lim, W.A.; Weissman, J.S.; Qi, L.S. CRISPR Interference (CRISPRi) for Sequence-Specific Control of Gene Expression. *Nature Protocols* **2013**, *8*, doi:10.1038/nprot.2013.132.
177. Cho, S.; Shin, J.; Cho, B.K. Applications of CRISPR/Cas System to Bacterial Metabolic Engineering. *International Journal of Molecular Sciences* 2018, *19*.
178. Lazar, S.; Prusty, M.R.; Bishara, K.; Sherman, A.; Fridman, E. RECAS9: Recombining Wild Species Introgression via Mitotic Gene Editing in Barley. *bioRxiv* **2020**.
179. Shlush, I. ben; Samach, A.; Melamed-Bessudo, C.; Ben-Tov, D.; Dahan-Meir, T.; Filler-Hayut, S.; Levy, A.A. Crispr/Cas9 Induced Somatic Recombination at the Crtiso Locus in Tomato. *Forests* **2021**, *12*, doi:10.3390/genes12010059.