Molecular Biology Reports (2022) 49:11443–11467
https://doi.org/10.1007/s11033-022-07741-2

ORIGINAL ARTICLE

CRISPR/Cas tool designs for multiplex genome editing and its applications in developing biotic and abiotic stress-resistant crop plants

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Received: 10 February 2022 / Accepted: 22 June 2022 / Published online: 24 August 2022

Abstract

Crop plants are prone to several yield-reducing biotic and abiotic stresses. The crop yield reductions due to these stresses need addressing to maintain an adequate balance between the increasing world population and food production to avoid food scarcities in the future. It is impossible to increase the area under food crops proportionately to meet the rising food demand. In such an adverse scenario overcoming the biotic and abiotic stresses through biotechnological interventions may serve as a boon to help meet the globe's food requirements. Under the current genomic era, the wide availability of genomic resources and genome editing technologies such as Transcription Activator-Like Effector Nucleases (TALENs), Zinc Finger Nucleases (ZFNs), and Clustered-Regularly Interspaced Palindromic Repeats/CRISPR-associated proteins (CRISPR/Cas) has widened the scope of overcoming these stresses for several food crops. These techniques have made gene editing more manageable and accessible with changes at the embryo level by adding or deleting DNA sequences of the target gene(s) from the genome. The CRISPR construct consists of a single guide RNA having complementarity with the nucleotide fragments of the target gene sequence, accompanied by a protospacer adjacent motif. The target sequence in the organism's genome is then cleaved by the Cas9 endonuclease for obtaining a desired trait of interest. The current review describes the components, mechanisms, and types of CRISPR/Cas techniques and how this technology has helped to functionally characterize genes associated with various biotic and abiotic stresses in a target organism. This review also summarizes the application of CRISPR/Cas technology targeting these stresses in crops through knocking down/out of associated genes.

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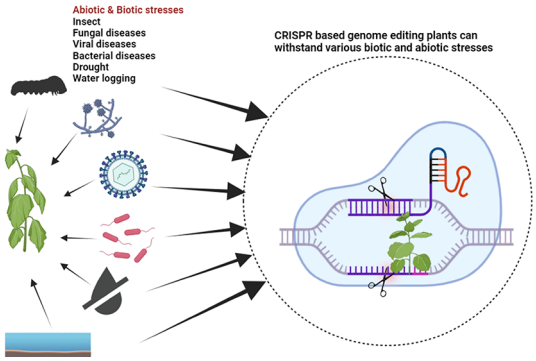
Graphical Abstract



Introduction

Seventy to eighty percent increase in food production is required to meet the ever-growing human population, which is estimated to increase to nine million by the middle of the 21st century. Plants are prone to various biotic and abiotic stresses, leading to significant reductions in plant yields. In addition to the growing population, extreme weather conditions, decreasing water availability, and agricultural land, such stresses are considerable limitations to food production. It takes enormous time and effort to have a plant with all the desirable traits through conventional breeding. Sometimes, it may be nearly impossible to land on a plant with all accumulative desirable traits due to these limiting factors. However, under such scenarios, genome editing (GE) technologies provide alternatives to inculcate desired traits into crops within a short period. These technologies are proving helpful in editing the genome of several crops that are difficult to be improved via conventional breeding. Thus, GE has emerged as powerful tool for plant breeding and functional genomics. Furthermore, technologies like CRISPR/Cas have helped acquire desirable traits and achieve functional characterization of specific genes.

Several kinds of mutations, including insertions, deletions, substitutions, replacement, and integration on the desired DNA sequence, have become possible with the help of SSNs (Sequence-Specific Nucleases). SSNs, in simple terms, are the molecular scissors capable of producing double-stranded breaks (DSBs) in the DNA, which are repaired by the repair mechanisms of the plant. The two repair mechanisms are NHEJ (Non- Homologous End Joining) and HDR (Homology Directed Repair), which results in insertions, deletions, or substitution mutations in the target region. NHEJ is error-prone, while HDR is more accurate as the former mechanism uses any sequence template to make repairs at DSB, leading to insertions and deletions at this location or the target sites. The latter is more accurate, as the template used in this mechanism needs to hold some homology to the target sequence. The major classes of SSNs include mega nucleases, ZFNs (zinc-finger nucleases), TALENs (TAL effector nucleases), and the most recent one, CRISPR/Cas (clustered regularly interspaced short palindromic repeats/ CRISPR-associated protein). In recent times, RNA-guided nucleases CRISPR/Cas have become the most preferred GE tool due to their ease of targeting the desired DNA sequence by manipulating the guide RNA sequence [1, 2].

Mechanism

The CRISPR system was discovered as a mechanism of adaptive immunity in bacteria, often to cleave foreign DNA sequences. CRISPR gained its name in 2002 based on its structure, described as a series of short palindromic repeats interspaced with short sequences. The CRISPR/Cas9 system consists of three components, i.e., tracer RNA (trRNA), CRISPR RNA (crRNA), and an endonuclease enzyme, Cas9. The tracer RNA (trRNA) and CRISPR RNA (crRNA) are together are called the guide RNA (gRNA). The bacteria use type II CRISPR to cut viral DNA and plasmids into small pieces and integrate them into their genome as CRISPR locus [3]. These integrated CRISPR sequences are later transcribed as gRNA during regular transcription, directing the endonuclease Cas9 to target foreign DNA based on sequence complementarity. The endonuclease makes a double strand break at the target site in the genome, which activates either NHEJ or HDR DNA repair mechanism in the cell [3]. NHEJ leads to gene knockout by insertions or deletions at the target site. In contrast, while repairing double strand breaks, HDR results in gene knocking as it uses donor segment homologous to the target site, making accurate substitution mutation at the target double strand break.

Cas endonucleases and engineered Cas9 variants

Cas9 endonuclease derived from *Streptococcus pyogenes* is the first characterized and most used endonuclease in genome editing. SpCas9 forms a ribonucleoprotein complex with sgRNA (single-guide RNA), which recognizes the target sequence adjacent to 5′-NGG-3′ PAM (Protospacer Adjacent Motif) and creates a double strand break [4]. *Sp*Cas9 has multiple domains, among which nucleic acid binding domains and nuclease domains are essential. The former helps *Sp*Cas9 to bind sgRNA and target DNA, while the nuclease domain consists of two HNH and RuvC domains, which cleaves at both the targeting and non-targeting strands of DNA, respectively [4]. The PI domain (PAM interacting domain) recognizes the PAM sequence

in the target organism's genome that helps bind the sgRNA and *Sp*Cas9 complex to the target. This requirement of the PAM sequence is a major limiting factor in CRISPR/Cas9 systems as it reduces the chance of selection of target sites [5]. Orthologous endonuclease or engineered variants of Cas9, such as Cpf1 from *Prevotella* and *Francisella*, commonly known as Cas12a, are used to overcome this limitation. Most importantly, Cas12a recognizes various PAM sequences and possesses only the RuvC domain, producing a staggering cut instead of a blunt one compared to Cas9 [6]. Cas12a recognizes T-rich PAM sequences such as 5′-TTTV-3′ or 5′-TTV-3′, which increases the possibilities of genome editing targets [7]. Cas12a is also promising as it requires only crRNA (~43 nt) instead of sgRNA (~100 nt) in the Cas9 system. Several orthologues of SpCas9 obtained from different bacteria used in CRISPR mediated genome editing are SaCas9 from *Staphylococcus aureus* [8], BlatCas9 from *Brevibacillus laterosporus* [9], and StCas9 from *Streptococcus thermophilus* [10] with PAM site as 5′-NNGRRT-3′, 5′-NNNNCNDD-3′ and 5′-NNAGAAW-3′, respectively. Moreover, *Sp*Cas9 has been engineered for PI domain generating variants recognizing alternative PAM sequences such as VQR, EQR, and VRER, which further recognize 5′-NGAN-3′, 5′-NGAG-3′ and 5′-NGCG-3′ PAMs, respectively [11, 12]. Cas9 has also been manipulated for nuclease domains and converted to Cas nickase by activating HNH or RuvC-like nuclease domains or dead Cas9 (dCas9) with only DNA binding ability. Furthermore, the base editors are fused with dCas9 to edit the target DNA by irreversibly converting one base pair to another without any cleavage on DNA strands [13]. The base editors such as CBEs (cytosine base editors), ABEs (adenine base editors), and RNA base editors are fused with dCAS9 or nickase Cas9 (nCas9) and used for precise editing to generate SNPs or stop codon [14].

Multi-targeting genome editing approaches

One of the primary advantages of CRISPR is multiplex editing, often used to edit multiple targets in the genome. Among the two approaches for multiplex editing, the first one uses a single promoter to express multiple sgRNA as a single transcript, while the second approach uses an individual promoter for each sgRNA [15]. This section provides a detailed discussion on the strategies used for multi-targeted genome editing.

tRNA mediated multiplex genome editing

The tRNA is the fundamental unit in each living cell, playing a pivotal role in translation. The tRNA processed from its primary transcript by RNA-processing machinery contains RNaseZ and RNaseP. The shorter sequences and self-splicing capacity of tRNA promoters make it suitable for transcribing the short and noncoding gRNA sequence used to separate multiplexed gRNAs in plants [15]. The synthesized tRNA-gRNA tandem sequences release individual sgRNA using endogenous RNA-processing machinery (Fig. 1). The RNaseZ and RNaseP make cleavage at the tRNA's 5,' and 3' ends, releasing the sgRNA from the transcript after processing [16]. Multiple sgRNA expression cassettes were designed using this technique to successfully edit 46 target sites in rice with 85% homologous heritable mutations in the target genes [17].

Another method with multiple gRNA uses dimeric RNA-guided FokI nucleases (RFNs), which do not require any specific sequence at 5' end for editing the target. The dimeric RFNs nuclease is considered better than the existing monomeric nucleases such as Cas9, which induce many off-target mutations. Additionally, the dimeric RFNs are target-specific as they use two gRNA to bind to the target, compared to the monomeric Cas9 nucleases [18]. The tRNA mediated strategy is used to edit different loci or delete the short fragments from the genome and the Cas9 fused transcription activator or repressor can control the expression of different genes [19]. Even though tRNA mediated systems are the best available for multiplex genome editing, a considered limitation is that this system does not work well for more than six gRNAs due to retarded editing efficiency. The efficacy of this technique has been evaluated for efficiency by using different promoters such as the SlEF1α promoter, which generated specific mutation with a low off-target mutation in rice protoplast editing.

Csy4 nuclease mediated multiplex genome editing

Another system used for multiplex genome editing is Csy4 endoribonuclease-mediated genome editing. This technique uses Csy4 obtained from the bacteria *Pseudomonas aeruginosa* and releases individual gRNA from multiple gRNA transcripts [16]. The construct for this system is designed using a tandem array of gRNA and restriction sites for Csy4 (Fig. 2). The bacterial origin Csy4 is cloned in the same vector and is transcribed into a host enzyme to release individual gRNA [20]. This strategy does not use host RNA machinery to excise gRNA; instead, it recognizes and binds the stem loop structure of RNA sequence 5'-GTTCACTGCCGTATAGGCAGCTAAGAAA-3' [21] and cuts at 20th position after guanine. The Csy4 and t-RNA systems used to generate deletions in 6 genes using 12 gRNAs from a single transcript have shown 100% higher efficiency in mutation induction than individual RNA polymerase III promoters [22]. This strategy of PTG (polycistronic tRNA-gRNA) and

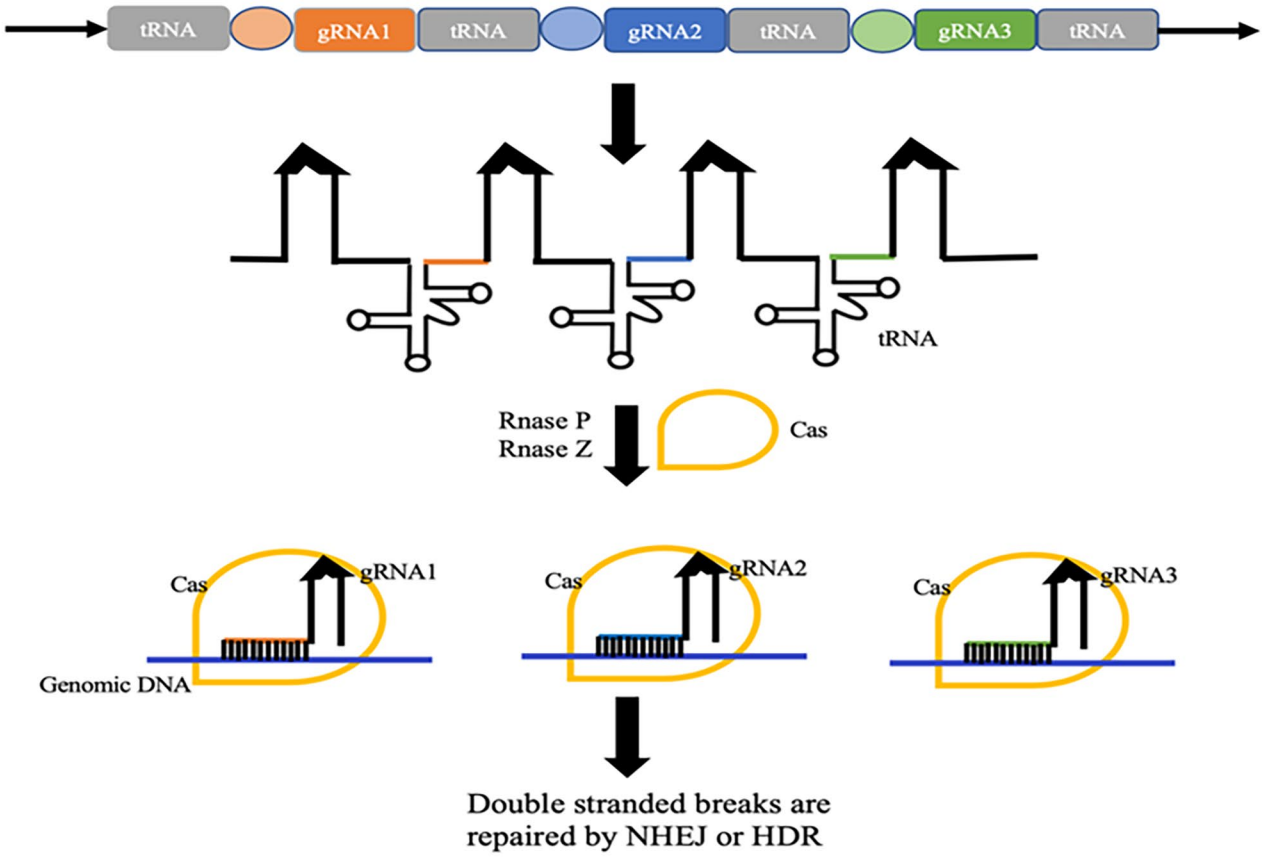


Fig. 1 tRNA mediated multiplex genome editing. The gray box represents the nucleic acid fragment for tRNA. The orange, blue and green box represent gRNA1,2, and 3 respectively. Cas nuclease is represented by a yellow shape. RNase P and RNase Z work with Cas endonuclease to cleave the tRNA releasing gRNA from the transcript after processing. (Modified from Xie et al 2015)

Csy4 is further used and validated in tobacco, wheat, Medicago, and tomato [22].

Drosha-based multiplex genome editing

The Drosha-based multiple targeted genome editing approach not explored much in plants uses a tandem array of gRNA-micro RNA (miRNA) (Fig. 3) or short hairpin (shRNA), expressed with a polymerase II promoter. Polymerase III promoters are often used to express sgRNA as they do not possess a 5' cap or 3' tail, but these are least recommended due to their short life [22]. Pol II is preferred in such cases as it expresses itself in a tissue-specific and flexible manner. Still, its redundant nuclease activity due to the 5' cap is a matter of concern. This situation is addressed by using a mi-RNA based strategy, which uses dROSHA (an RNase III enzyme) to excise gRNA and miRNA.

CRISPR/Cas against biotic stress in plants

CRISPR/Cas for fungal disease resistance in plants:

The CRISPR/Cas9 genome editing technology has been explored by various scientists and researchers in the field of plant pathology. This technique was used to enhance the resistance against blast disease in rice by manipulating the transcription factor *OsERF922*. C-ERF922 induced plants were identified for target mutation in the *OsERF922* gene without transferring DNA in T_1 and T_2 generations. The results were auspicious as all selected mutant lines showed significant enhancement in rice blast resistance compared to wild type at both seedling and tillering stage when inoculated with *M. oryzae* [23]. Further, the technique was explored in the plant defense system involving the endocytic and exocytic vesicle trafficking in relation to pathogen infection. The exocyst is an octameric conserved protein complex involved in exocytosis for tethering vesicles to membranes. *OsSEC3A* is considered an essential part of the exocyst complex in paddy, and mutation in this gene

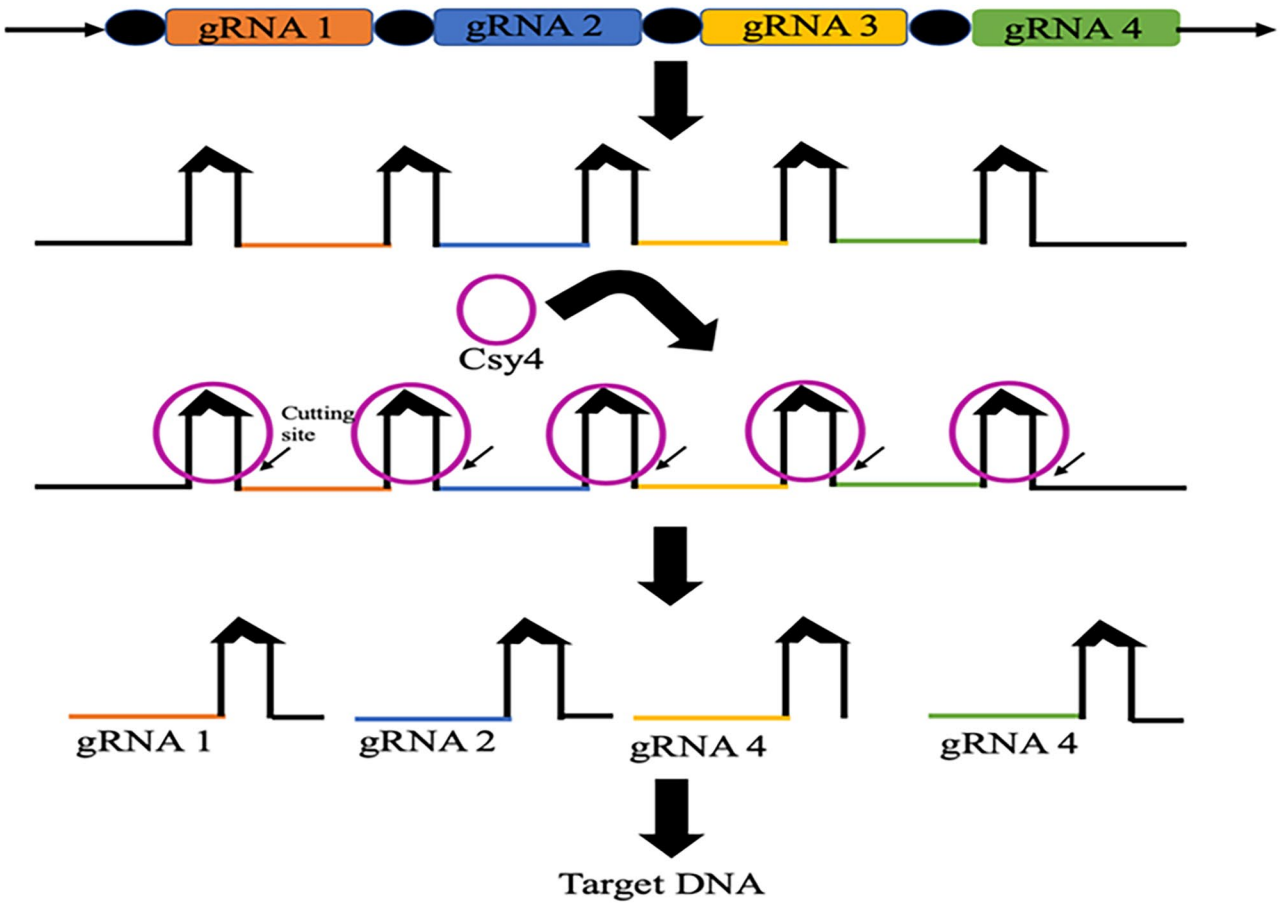


Fig. 2 Csy4 nuclease mediated multiplex genome editing. The orange, blue, yellow, and green box represents gRNA1,2,3, and 4 respectively. Csy endonuclease is represented by purple oval shape. Csy4 is used to release different gRNAs from multiple transcripts. (Modified from Ferreira et al 2018)

by sgRNA resulted in reduced plant height and induced cell death seen in the form of lesion-mimic. The *ossec3a* plant showed upregulation of PR and SA biosynthesis genes resulting in resistance to rice blast pathogen [23]. This study stated that OsSEC3A provides the resistance against blast disease of rice but at the cost of energy used for plant growth and development. CRISPR/Cas9 technology is also used to develop disease-resistant male sterile lines for hybrid rice breeding programs. Sequence-specific mutations in *TMS5*, *Xa 13* & *Pi 21* genes via CRISPR/Cas9 yielded the thermo-sensitive male sterile lines with resistance to bacterial blight and blast of rice [24].

The technique was then explored in other fungal pathogen systems owing to its promising results. Knocking down of *TaEDR1* gene of wheat via RNA interference stated its negative role in enhanced resistance against powdery mildew caused by *Blumeria graminis*. It was found that plants with homologous mutants of *TaEDR1* generated by CRISPR/Cas9 showed enhanced resistance against powdery mildew pathogen infection, and no off-target mutations were observed in genetically edited plants [25]. Similarly, heritable resistance against powdery mildew was produced when all the three homoalleles of *TaMLO* encoding for Mildew Resistance Locus proteins were edited using TALENs and then *TaMLO-A1* allele was edited using CRISPR/Cas9 in the same plant [26].

The reduced mycorrhizal colonization (rmc) mutants of the tomato plant produce a phenotype without mycorrhizal colonization in roots and are susceptible to *Fusarium* wilt. This phenotype arises from losing a gene similar to *CYCLOPS*. The *rmc* mutants consist of chromosomal deletion, which interferes with the sequences of five genes. Deletion in the gene *Solyco8go75770* expressed in the roots encoding for a transmembrane protein showed susceptibility to *Fusarium* wilt [27]. At the same time, the putative complementation lines were similar to wild type, conferring *Fusarium* wilt tolerance. The major advantage of CRISPR/Cas9 over conventional breeding or transgenic

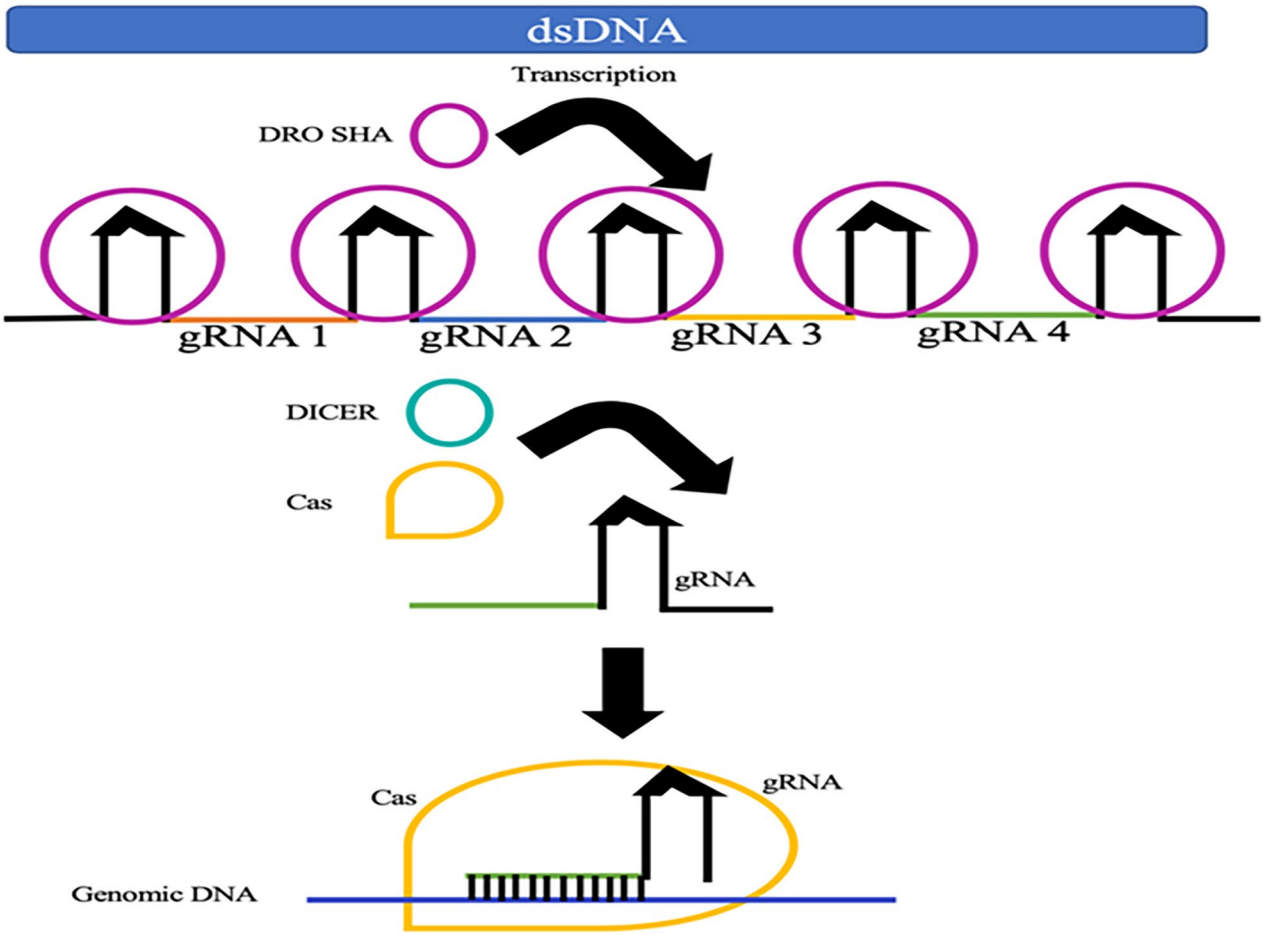


Fig. 3 DROSHA based multiplex genome editing. The orange, blue, yellow, and green box represents gRNA1,2,3, and 4 respectively. DRO SHA, DICER, and Cas endonuclease are represented by purple, blue, and yellow shapes respectively. Different gRNAs are separated from each other by DRO SHA. (Modified from Xie et al 2015)

crop development is the time, as it helps develop resistant varieties way faster than conventional breeding. Tomelo, a tomato variety resistant to powdery mildew, was developed using the CRISPR/Cas9 technique within eight months by targeting *SlMLO1* using double sgRNA target strategy [28]. The whole-genome sequence of the Tomelo variety showed no transgene insertion and no off-type mutations. Earlier studies reveal that RNAi's (RNA-interference technology) silencing of a susceptibility gene, *PMR4*, in tomato plants enhances the resistance against powdery mildew. Recently, CRISPR/Cas9 was used to completely knock-down the *PMR4* gene using four single gRNAs to make large mutations in the *PMR4* locus. The deletions, insertions, and inversions in the *PMR4* locus resulted in the reduced susceptibility of the mutant plants to powdery mildew [29].

Defense signaling pathways against biotic and abiotic stress involve various mitogen-activated protein kinases (MAPKs). CRISPR/Cas9 technique was used to investigate the role of *SlMAPK3* locus in resistance to *Botrytis cinerea* in tomato plants with *slmapk3* mutant plants showing high susceptibility to *B. cinerea*. Results suggested positive role of *SlMAPK3* in resistance against *B. cinerea* through ROS (Reactive Oxygen Species) production, SA (Salicylic acid), and JA (Jasmonic acid) signaling pathways [30] (Table 1).

CRISPR/Cas for bacterial disease resistance in plants:

The *Xanthomonas oryzae* pv *oryzae*- (Xoo) rice system is well studied and used as a model for studying various susceptible genes (SWEET genes) in monocots. Transcription activator-like Effectors (TALEs) secreted by Xoo strains through Type III effectors interact with EBEs (effector binding elements) in promoter regions of *SWEET* genes to induce expression of genes for the production of sucrose that makes plants susceptible. The CRISPR/Cas9 mediated knockout of *SWEET* genes generated bacterial blight resistant plants [31]. The genome editing of the promoter of *SWEET13*, the target gene of *PthXo2* (TAL effector of

Table 1 Application of CRISPR/Cas based genome editing tool for developing biotic stress resistant crop plants

Disease	Crop plant	Pathogen	Target Gene	Mutation	Phenotype	Nuclease	Transformation Method	Reference
Fungal Diseases								
Powdery Mildew	Wheat	*Blumeria graminis* f. sp. *tritici*	*TaMLO* homologs	Insertion and deletion (indels) mutations	Resistance to powdery mildew	Cas9	Biolistic transformation of wheat	[26]
Powdery Mildew	Wheat	*Blumeria graminis* f. sp. *tritici*	*TaEDR1*/cds region	Insertion and deletion (indels) mutations	Resistance to powdery mildew	Cas9	Biolistic transformation of wheat	[25]
Fusarium wilt	Tomato	*Fusarium oxysporum f. sp. lycopersici*	Solyc08g075770/ genomic region	Insertion and deletion (indels) mutations	Enhanced susceptibility to disease	Cas9	Agrobacterium-mediated transformation method	[27]
Powdery Mildew	Tomato	*Oidium neolycopersici*	*PMR4*	Insertion, deletion and inversion mutations	Enhanced susceptibility to disease	Cas9		[30]
Gray mold	Tomato	*Botrytis cinerea*	*SlMAPK3*		Enhanced susceptibility to disease	CRISPR/Cas9 system		[30]
Rice blast	Rice	*Magnaporthe oryzae*	OsERF922/cds region	Insertion and deletion (indels) mutations	Enhanced resistance to disease	Cas9	Agrobacterium-mediated transformation method	[23]
Phytophthora blight	Tomato	*Phytophthora capsici*	SIDMR6–1/cds region	Deletions	Enhanced disease resistance	Cas9		
Rice blast	Rice	*Magnaporthe oryzae*	Pi21/cds region OsMPK5/ cds region	Insertion and deletion (indels) mutations	Enhanced disease resistance	Cas9	n.d.	[24]
Rice blast	Rice	*Magnaporthe oryzae*	OsMPK5/cds region	Insertion and deletion (indels) mutations	Disease resistance not confirmed	Cas9	n.d.	[47]
Powdery mildew	Tomato	*Oidium neolycopersici*	SlMlo1/cds region	Deletions mutations	Enhanced resistance to disease	Cas9	Agrobacterium-mediated transformation method	[28]

Table 1 (continued)

Disease	Crop plant	Pathogen	Target Gene	Mutation	Phenotype	Nuclease	Transformation Method	Reference
Powdery mildew	Tomato and Arabidopsis	*Oidium neolycopersici*	PMR4/cds region	Deletion and inversion mutation	Enhanced resistance to disease	Cas9	Agrobacterium-mediated transformation method	[29]
Fusarium wilt	Watermelon	*Fusarium oxysporum fsp niveum*	ClPSK1	Deletions mutations	Enhanced resistance to disease	Cas9	Agrobacterium-mediated transformation method	[48]
Powdery mildew	Grapes	*Erysiphe necator*	MLO-7/cds region	Insertion and deletion (indels) mutations	Resistance to disease developed	Cas9	Biolistic transformation of wheat	[49]
Black pod disease	Cacao	*Phytophthora tropicalis*	NPR3	Insertion and deletion (indels) mutations	Enhanced resistance to disease	Cas9	Agrobacterium-mediated transformation method	[50]
Phytophthora fruit rot and root rot	Papaya	*Phytophthora palmivora*	alEPIC8/cds region	Deletion mutation	Increased resistance against *Phytophthora palmivora*	Cas9	Agrobacterium-mediated transformation method	[51]
Corn smut	Maize	*Ustilago maydis*	bW2 and bE1	Deletion mutation		Cas9	Agrobacterium-mediated transformation method	[52]

Table 1 (continued)

Disease	Crop plant	Pathogen	Target Gene	Mutation	Phenotype	Nuclease	Transformation Method	Reference
Verticillium wilt	Cotton	*Verticillium dhaliae*	Gh14-3-3d	Deletion mutation	Enhanced resistance to *Verticillium dhaliae*	CRISPR/Cas9	Agrobacterium-mediated transformation method	[53]
Bacterial Diseases								
Fire blight	Apple (*Malus pumila*)	*Erwinia amylovora*	DIPM-1, DIPM-2, and DIPM-4	Insertions and deletions (indels) mutations	DIPMs exhibit direct physical interaction with the disease-specific gene of Erwinia amylovora, which may act as a susceptible factor	CRISPR/Cas9 RNPs system	Biolistic transformation of wheat	[49]
Bacterial speck	Tomato	*Pseudomonas syringae* pv. *tomato* (Pto) DC3000	SlJAZ2/cds region	Insertions, deletions, and substitutions	Enhanced disease resistance, defence trade-off solved	CRISPR/Cas9	Agrobacterium-mediated transformation method	[36]
Bacterial spot	Tomato	*Xanthomonas* spp.	SIDMR6–1/cds region	Deletions	Enhanced disease resistance	CRISPR/Cas9	Agrobacterium-mediated transformation method	[36]
Citrus canker	Wanjincheng orange (Citrus sinensis Osbec)	*Xanthomonas citri* subsp. *citri*	Gene CsLOB1 (LATERAL ORGAN BOUNDARIES1) promoter	Insertions, deletions, and substitutions	Mutant plants showed tolerance against citrus canker	CRISPR/Cas9 system	Agrobacterium-mediated epicotyl transformation method	[37]

Table 1 (continued)

Disease	Crop plant	Pathogen	Target Gene	Mutation	Phenotype	Nuclease	Transformation Method	Reference
Bacterial Blight	Rice	*Xanthomonas oryzae* pv. *oryzae*	SWEET14/promoter	Insertions and deletions	Enhanced broad-spectrum disease resistance	CRISPR/Cas9	Agrobacterium-mediated transformation method	[35]
Bacterial Blight	Rice	*Xanthomonas oryzae* pv. *oryzae*	SWEET11, SWEET13 and SWEET14/promoter	Insertions and deletions	Enhanced broad-spectrum disease resistance	CRISPR/Cas9	Agrobacterium-mediated transformation method	[34]
Bacterial Blight	Rice	*Xanthomonas oryzae* pv. *oryzae*	Os8N3/promoter region	Insertions and deletions	Enhanced disease resistance	CRISPR/Cas9	Agrobacterium-mediated transformation method	[33]
Bacterial Blight	Rice	*Xanthomonas oryzae* pv. *oryzae*	OsSWEET11 and OsSWEET14/promoter region	Insertions and deletions	Enhanced broad-spectrum disease resistance	CRISPR/Cas9	Agrobacterium-mediated transformation method	[54]
Bacterial Blight	Rice	*Xanthomonas oryzae* pv. *oryzae*	Xa13/cds region	Insertions and deletions	Enhanced disease resistance	CRISPR/Cas9	Agrobacterium-mediated transformation method	[24]
Bacterial Blight	Rice	*Xanthomonas oryzae* pv. *oryzae*	OsSWEET11, OsSWEET14/promoter	Insertions and deletions	Disease resistance not confirmed	CRISPR/Cas9	Agrobacterium-mediated transformation method	[31]

Table 1 (continued)

Disease	Crop plant	Pathogen	Target Gene	Mutation	Phenotype	Nuclease	Transformation Method	Reference
Bacterial Blight	Rice	*Xanthomonas oryzae* pv. *oryzae*	OsSWEET13/cds region	Insertions and deletions	Enhanced disease resistance	CRISPR/Cas9	Agrobacterium-mediated transformation method	[32]
Bacterial Panicle Blight	Rice	*Burkholderia glumae*	OsMPK5/cds region	Insertions and deletions	Disease resistance not confirmed	CRISPR/Cas9	Agrobacterium-mediated transformation method	[47]
Viral Diseases								
DNA viral disease	Tobacco	CLCuMuV	IR and C1	In-dels	Complete resistance to CLCuMuV	CRISPR/Cas9	Agrobacterium-mediated transformation method	[55]
	Banana	BSV	eBSV Sequence	In-dels	Inactivation of eBSV gave asymptomatic plants	CRISPR/Cas9	Agrobacterium-mediated transformation method	[56]
	Barley	WDV	MP, CP, Rep/Rep, IR	Insertion	No disease symptoms and virus presence	CRISPR/Cas9	Agrobacterium-mediated transformation method	[57]
	Tobacco	TYLCV, BCTV, MeMV	IR, CP, RCRII	In-dels	No disease symptoms and delayed or reduced virus accumulation	CRISPR/Cas9	Agrobacterium-mediated transformation method	[38]

Table 1 (continued)

Disease	Crop plant	Pathogen	Target Gene	Mutation	Phenotype	Nuclease	Transformation Method	Reference
	Tobacco	BeYDV	LIR, Rep	In-dels	Reduced virus load and symptoms	CRISPR/Cas9	Agrobacterium-mediated transformation method	[58]
	Tobacco and Arabidopsis	BSCTV	IR, CP, Rep	In-dels	Geminivirus-resistant plants of both Tobacco and Arabidopsis	CRISPR/Cas9	Agrobacterium-mediated transformation method	[45]
	Tobacco	CLCuKoV, TYLCV, TYLCSV, MeMV, BCTV-Logan, BCTV-Worland	IR, CP, Rep	In-dels	Disease resistance not confirmed	CRISPR/Cas9	Agrobacterium-mediated transformation method	[59]
	Tobacco and Tomato	TYLCV	CP, Rep	In-dels	Disease resistance developed	CRISPR/Cas9	Agrobacterium-mediated transformation method	[60]
	Cassava	ACMV	DNA- A	In-dels	Fail to confer effective resistance	CRISPR/Cas9	Agrobacterium-mediated transformation method	[61]
RNA viral diseases	Tomato	PVX, TMV, TMV	DCL2	In-dels	Mutants showed viral symptoms when injected by targeted viruses	CRISPR/Cas9	Agrobacterium-mediated transformation method	[62]

Table 1 (continued)

Disease	Crop plant	Pathogen	Target Gene	Mutation	Phenotype	Nuclease	Transformation Method	Reference
	Cucumber	CVYV, ZYMV, PRSV-W	elf4E/cds region	Deletions	Resistance to CVYV, ZYMV and PRSMV	CRISPR/Cas9	Agrobacterium-mediated transformation method	[40]
	Arabidopsis	TuMV	Elf(iso)4E/cds region	In-dels	Potyvirus resistant plants	CRISPR/Cas9	Agrobacterium-mediated transformation method	[42]
	Cassava	CBSV	nCBP-1 and nCBP-2/cds region	In-dels	Reduced virus load and symptoms	CRISPR/Cas9	Agrobacterium-mediated transformation method	[41]
	Tobacco and Arabidopsis	CMV	ORF1a, ORFCP and 3'-UTR	no cleavage	Reduced virus load and Symptoms	CRISPR/Cas9 (FnCas-9CRISPR/Cas9 (FnCas9)	Agrobacterium-mediated transformation method	[63]
	Tobacco	TuMV	GFP, Hc-pro and CP	n.d.	Reduced virus load and appearance	Cas13a	Agrobacterium-mediated transformation method	[64]
	Rice	RTSV	eIF4G			Cas13a	Agrobacterium-mediated transformation method	[65]

Xoo), generated the mutant plants showing resistance to Blb [32]. Xoo strains containing *PthXo1* target the *Os8N3* gene to activate sugar transporters to make nutrients available for the growth and multiplication of pathogens. The studies suggested that knockdown of *Os8N3* enhanced resistance to Xoo but exhibited abnormal pollen development. The rice plants with the homologous knockout mutant of EBEs of the *Os8N3* gene via CRISPR/Cas9 exhibited significant resistance to Xoo with no fitness cost, including pollen development [33]. The CRISPR/Cas9 system was also

used to introduce five simultaneous mutations in the promoter region of *SWEET13* and *SWEET14* genes of rice lines Kitaake and japonica, and rice varieties IR64 and Ciherang-Sub1. The homologous mutant plant displayed robust broad-spectrum resistance to most of the Xoo strains [34]. The CRISPR/Cas9 mediated genome editing was made in super basmati rice to mutate 4 EBEs (Effector Binding Elements) in the promoter region of the *SWEET14* gene, which resulted in resistance against their specific TALEs (AvrXa7, PthXo3, and TalF) of Xoo strains [35].The bacterial speck disease of tomato by *Pseudomonas syringae* produces coronatine (COR) to imitate the reopening of stomata for bacterial infection. AtJAZ2 is the receptor of COR, which signals stomatal opening. The mutant of *AtJAZ2* was generated by CRISPR/Cas9 genome editing (AtJAZ2Δ), which acted as the repressor of stomatal reopening via COR and provided resistance against bacterial speck [36].

Apart from numerous uses in field crops, CRISPR/Cas has also made its way in addressing biotic and abiotic stresses in horticultural crops. The PthA4 effector molecule of *Xanthomonas citri* sub. spp. *citri* interacts with EBEs of promoter regions of the *CsLOB1* gene making Duncan grapefruit susceptible to citrus canker. Type1 allele of *CsLOB1* disrupted by designing gRNA targeting its promoter region failed to impart resistance against canker. However, five pCas9/CsLOB1sgRNA constructs designed to mutate promotors of both alleles in Wanjincheng orange successfully imparted resistance in homologous mutant lines against Xcc strains [37].

CRISPR/Cas9 for Plant viral disease management

CRISPR/Cas9 is a highly target-specific, powerful molecular immunity system to address different problems caused by viruses. Current studies have verified and demonstrated the utility and efficiency of this system. *Nicotiana benthamiana* plants expressing CRISPR/Cas9 displayed resistance against *Beet curly top virus*, *Merremia mosaic virus*, and *Tomato yellow leaf curl virus* showing degradation and introduced mutations at target sequences [38]. The Cas9 transformed tobacco has been developed via agroinfiltration method using *Tabacco rattle virus* (TRV) that carries an expression cassette of different sgRNAs. This system is now used as an antiviral tool to suppress many DNA viruses, particularly by cleaving the DNA from specific regions. Concluding this, the sgRNAs not only exhibit interference activity but can target important genomic regions of the virus, such as the origin of replication (OR) in the intergenic region (IR) and movement proteins of the DNA virus. *N. benthamiana* plants edited with this system show significantly attenuating or abolishing symptoms of infection only because of deferred viral DNA. Subsequently, co-delivery of multiple sgRNAs using the *Tobacco rattle virus* (TRV) system have an additive outcome, resulting in higher interference levels than those attained using single sgRNAs. Targeting IR and coat protein (CP) using separate RNA2 genomes reduced viral accumulation and replication similar to the levels obtained by targeting either CP or IR via single sgRNA [38]. Hence, it is possible to target multiple DNA viruses using a single sgRNA only by targeting a conserved sequence that precedes the PAM sequence. By means of multiple sgRNAs, the ability for multiplexed editing of single or multiple viruses can be achieved. CRISPR/Cas9 system holds the potential to overcome resistance problems by targeting newly evolved viral strains via new sgRNAs that apply to all plant DNA viruses. Geminivirus-based VIGE (virus induced gene editing) is a powerful tool in genome editing and is being used to precisely target plant genome locations and cause several mutations [39]. Direct delivery and feasibility of virus-mediated Cas9/sgRNA using the *Cabbage Leaf Curl virus* has been demonstrated using modified *Cabbage Leaf Curl virus* (CaLCuV). VIGE is performed to express gRNAs in plants that can express Cas9 protein. The modified CaLCuV vector (VIGE of *NbPDS3* and *NblspH*) has been used to express gRNAs and edit target genes resulting in very high mutation rates with the photobleached phenotype of a newly developed plant. Some subviral RNA pathogens depend on non-coding helper viruses for their spread and replication are known as satellite RNAs (siRNAs). In *N. tabacum*, complete transcriptional repression of β glucuronidase (GUS) transgene that was fused with Y satellite RNA sequence (35 S::GUS:Sat) of *Cucumber mosaic virus* (CMV) resulted in suppression due to specific DNA methylation at Y-Satellite RNA sequence compared to 35 S-GUS transgene with no Y-Sat sequence [39]. CRISPR/Cas9 system has been used for broad-spectrum resistance targeting and disrupting translation initiation like factors *eIF4E* gene without affecting the plant genome in cucumber. Immunity was exhibited against the family Potyviridae, mainly *Cucumber vein yellowing virus* (CVYV), *Zucchini yellow mosaic virus* (ZYMV), and *Papaya ring spot mosaic virus*-W (PRSV) by introducing small deletions and SNPs in recessive *eIF4E* gene in T1 generation of cucumber [40]. *Cassava brown streak virus* (CBSV) is a major constraint for Central and Eastern Africa cassava yields. The viral genome-linked protein (VPg) interacts with novel cap-binding protein-1 and 2 (NCBP-1/NCBP2). However, the virus showed delayed and reduced symptoms when the double mutants of *ncbp-1/ncbp2* were generated using the CRISPR/Cas9 system, further reducing the severity and incidence of root necrosis [41]. Recessive resistance alleles are identified against various Potyviruses, including *eIF4E*, and its paralogue, *eIF(iso)4E*. In *Arabidopsis thaliana*, using sequence-specific deleterious point

mutations at *eIF(iso)4E* locus, complete resistance against *Turnip Mosaic Virus* (TuMV) was attained with no effect on plant vigor [42]. Geminiviruses are being used as vectors for genome editing because they can infect a wide range of crops like wheat, cotton, maize, tomato, beans, legumes, and some ornamental plants. These viruses require only a single protein to replicate (replication-associated protein; REP) inside the host cells to produce lots of sequence-specific nucleases, significantly increasing the target efficiency. The efficiency in modifying the tomato genome using geminivirus replicons had tenfold higher frequencies than the conventional *Agrobacterium* mediated DNA delivery method. In *Solanum tuberosum* also, the geminivirus replicon was used to deliver SSNs in *ACETOLACTATE SYNTHASE1* (ALS1). The repair templates were generated within the ALS1 locus to incorporate herbicide inhibiting point mutations, which resulted in reduced herbicide susceptibility in the phenotype [43]. Different kinds of grasses belonging to the family *Poaceae* exhibit several pathogenic attacks and are thus harder to transform and make transgenic. Studying hexaploid wheat and determining high-throughput gene targeting using CRISPR/Cas9 and DNA replicons, a 110-fold upsurge in the expression of a reporter gene was acquired using a deconstructed form of the *Wheat dwarf virus* (WDV). WDV infects a variety of grasses, including most cereals, and has been previously used to express foreign proteins in wheat and maize cells [22]. Knowledge and technology that enable exact and efficient DNA substitution or knock-in, lately referred to as KI can transform crop generation by accuracy in plant molecular breeding. In rice, no geminivirus-based genome editing has been established, and reported DSBs produced by merging CRISPR/Cas9 and geminiviral vectors accomplished up to 19.4% targeting KI frequency. In molecular rice breeding, an efficient KI method has been developed using WDV-derived targeted KI system, making it a simpler and more efficient device for transferring copious donor DNA into rice cells [44]. *Beet severe curly top virus* (BSCTV) accumulation hinders when sgRNA-Cas9 constructs are introduced to the target region in *N. benthamiana* and *Arabidopsis.* Introduction of plasmids, pHSN401-A7 into *N. benthamiana*, and pHSN401-C3 in *Arabidopsis* using *Agrobacterium*-mediated transformation targeting three different regions (A7, B7, and C3) resulted in reduced virus accumulation by 65%, 66%, and 70%, respectively and generated virus-resistant plants without any off-target costs [45]. Off-target mutations might occur due to the extended expression of Cas9 nuclease and the tolerance of sgRNA sequence mismatches. Thus, this virus-inducible genome editing system could be used in engineering virus-resistant plants without off-target effects. Apart from DNA viruses, RNA viruses also contribute significantly to crop losses. To address these losses, more CRISPR/Cas systems have been developed from other bacteria such as Cas13a from *Leptotrichia shahii* (LshCas13a) and the Cas9 from *Francisella novicida* (FnCas9) [7]. FnCas9 was used for the first time in *Nicotiana* and *Arabidopsis*, targeting CMV and TMV, reducing their accumulation and disease symptoms. The LshCas13a system can target different RNA viruses, including dsRNA genomes and +/-ssRNA virus. This system was used to cleave Rice stripe mosaic virus (RSMV) and the genomic RNA of Southern rice black-streaked dwarf virus by overexpressing crRNA-LshCas13a, specifically targeting the viral genome in rice plants [46]. Above mentioned studies prove that both DNA and RNA viruses have less chance to resist, overcome and escape the CRISPR/Cas antiviral system by mutating their genomes, creating stable and less heritable off-target effects.

CRISPR/Cas against abiotic stresses in plants:

Climate change is posing a threat to food security and agriculture. It is particularly more important in the tropical regions, especially Africa and South Asia, which already suffer from substantial food insecurity due to the detrimental effects of climate change [66]. With the rapid increase in the human population, which is predicted to reach 9.7 billion by 2050, global temperatures are also set to rise significantly. Even slight increases in average temperature, as little as 1 °C, may lead to a reduction in grain yields of rice, wheat, and maize by 5–10%, 6–12%, and 20–30% respectively, potentially weakening our food stocks in a fast-growing population [67]. It is difficult for us to control the population increase as plant scientists. However, we possess the capabilities to develop climate-resilient crop varieties that can thrive and flourish under such challenging conditions. These varieties must sustain harsh climatic conditions such as drought, floods, heat, cold, or heavy metal stresses. This requires a search for new and diverse germplasm, which historically performs well either through discoveries in natural variations or by selective breeding [68–70]. The other possibility is the creation of the mutant populations that are screened to search for new sources of the variations, the novel beneficial mutations can in turn be included in breeding programs. Modern genome editing technology tools like CRISPR enable the user to introduce desirable genomic changes accurately in almost all the crops plants and thus showing enormous potential as a tool for creating novel climate-resistant crops.

During the last decade, there has been a rapid development in gene-editing techniques with the CRISPR/Cas system. This study aims to provide broader coverage of the

Table 2 Application of CRISPR/Cas based genome editing tool for developing abiotic stress resistant crop plants

Trait	Crop plant	Target Gene	Mutation	Phenotype	Nuclease	Transformation Method	Reference
Drought							
	Arabidopsis	*OST2*	New allele created	Altered stomata closing time	Cas9	*Agrobacterium tumefaciens*	[93]
	Maize	*ARGOS8*	Deletion in the promoter and 5'-UTR	Increase ethylene responses and yield under droughts	Cas9	Particle bombardment	[71]
	Rice	*SAPK2*	Mutation in the third exon	ABA insensitive phenotype, more sensitivity to drought and reactive oxygen species	Cas9	*Agrobacterium tumefaciens*	[70]
	Rice	*OsEBP89*	Knockout of gene	Increase the drought tolerance ability of plant	Cas9	*Agrobacterium tumefaciens*	[72]
	Tomato	*SlMAPK3*	Knockout of the third exon	Higher wilting, increase hydrogen peroxide, and low antioxidant production	Cas9	*Agrobacterium tumefaciens*	[73]
	Tomato	*SlNPR1*	Small insertions and deletions	Aids in plants ability to tolerate the drought	Cas9	*Agrobacterium tumefaciens*	[74]
	Wheat	*TaDREB2*	Small insertions and deletions	Activation of dehydrating responsive element binding protein	Cas9	Protoplast transfection	[88]
Salinity							
	Rice	*OsRR22*	Small insertions, deletions, and substitution	Enhancement of salinity tolerance	Cas9	*Agrobacterium tumefaciens*	[46]
	Soybean	*Drb2a, Drb2b*	In-frame, frame-shift and double homozygous mutant	Increase in salinity and drought tolerance	Cas9	*Agrobacterium tumefaciens*	[75]
Heavy Metal							
	Arabidopsis	*CrRLK1L*	Frameshift and nonsense mutation in each gene	Aid in hypocotyl elongation and root growth under nickel, cadmium and zinc	Cas9	*Agrobacterium tumefaciens*	[92]
	Arabidopsis	*AtHMA4*	Knockout of gene	Increase zinc tolerance of plants	Cas9	*Floral dip transformation*	[93]
	Rice	*OsHAK1*	Knockout of gene	Inactivation of transportation of cesium to grain	Cas9	*Agrobacterium tumefaciens*	[94]
	Rice	*OsNramp5*	Small insertions and deletions	Decrease in transporter of cadmium to roots and shoots, with no effect on grain yield	Cas9	*Agrobacterium tumefaciens*	[76]
	Rice	*OsARM1*	Insertion in promoter region	Increase tolerance to high arsenic	Cas9	*Agrobacterium tumefaciens*	[78]
	Rice	*OsLCT1*	Single insertion/deletion to deletion of fragment upto 49 nucleotides	Less accumulation of cadmium in the grain without impact on grain yield	Cas9	*Agrobacterium tumefaciens*	[77]
	Rice	*OsPRX2*	Small insertions and deletions	Potassium deficiency tolerance increases in the plant	Cas9	*Agrobacterium tumefaciens*	[95]
	Rice	*OsATX1*	Knockout of gene	Interfere with transportation of copper to shoots and other reproductive parts	Cas9	*Agrobacterium tumefaciens*	[96]
Herbicide							
	Arabidopsis	*ALS*	Single base editing	Resistance to Tribenuron	Cas9	*Agrobacterium tumefaciens*	[97]
	Maize	*ALS1 and ALS2*	Single base editing	Resistant to Chlorsulfuron	Cas9	Biolistic delivery	[98]
	Chile pepper	*CaEPSPS*	Site directed mutagenesis	Resistant to Glyphosate		*Agrobacterium tumefaciens*	[80]
	Flax	*EPSPS*	Single base editing	Resistant to Glyphosate	Cas9	Polyethylene glycol mediated delivery	[99]

Table 2 (continued)

Trait	Crop plant	Target Gene	Mutation	Phenotype	Nuclease	Transformation Method	Reference
	Potato	*ALS1*	Point mutation	Resistant to Imidizolinone	Cas9	*Agrobacterium tumefaciens*	[43]
	Rice	*BEL*	Small insertions and deletions	Resistant to Bentazon and Sulfonylurea	Cas9	*Agrobacterium tumefaciens*	[100]
	Rice	*C287T*	Point mutation	Resistant to herbicide imazamox	dCAS9	*Agrobacterium tumefaciens*	[101]
	Rice	*OsEPSPS*	Replacement of the second exon	Resistant to Glyphosate	Cas9	Biolistic transformation	[79]
	Rice	*ALS*	Point mutation	Resistant to chlorsulfuron	Cas9	Particle bombardment	[82]
	Rice	*SF3B1*	Excision of different exons	Resistance to splicing inhibitors	Cas9	*Agrobacterium tumefaciens*	[102]
	Soybean	*ALS1*	Single nucleotide changes	Resistant to Chlorsulfuron	Cas9	Particle bombardment	[83]
	Tomato	*ALS*	Single nucleotide conversion	Resistant to Chlorsulfuron	Cas9	*Agrobacterium tumefaciens*	[81]
	Watermelon	*ALS*	Single nucleotide conversion	Resistant to Tribenuron	Cas9	*Agrobacterium tumefaciens*	[84]
	Wheat	*ALS*	Single nucleotide changes	Resistant to Nicosulfuron, Mesosulfuron, and Imazapic	Cas9	Particle bombardment	[103]
	Wheat	*ALS*	Single nucleotide changes	Resistant to Nicosulfuron	Cas9	*Agrobacterium tumefaciens*	[104]
Heat							
	Maize	*ZmTMS5*	Small insertions and deletions	Creation of thermosensitive maize lines for hybrid development	Cas9	Particle bombardment	[86]
	Rice	*PYL1, PYL2, PYL3, PYL4, PYL5, PYL6, and PYL12*	Small insertions and deletions	Greater high temperature tolerance, reduced preharvest sprouting, and increase grain yield	Cas9	*Agrobacterium tumefaciens*	[105]
	Rice	*TMS5*	Single nucleotide insertion and large deletions	Production of thermo insensitive genic male sterile lines for hybrid seed production	Cas9	*Agrobacterium tumefaciens*	[85]
	Tomato	*SlAGL6*	Knockout of the second exon	Ensure fruit production under heat stress	Cas9	*Agrobacterium tumefaciens*	[87]
	Wheat	*TaERF3, and TaDREB2*	Small insertions and deletions	Aids in activation wheat dehydration responsive element binding proteins	Cas9	Protoplast transformation	[88]
Cold							
	Rice	*OSAnn3*	Knockout of promoter	Decrease cold tolerance	Cas9	*Agrobacterium tumefaciens*	[90]
	Tomato	*slcbf1*	Small insertions and deletions	Increase the ability to tolerate chilling stress	Cas9	*Agrobacterium tumefaciens*	[89]
Flooding							
	Rice	OsEBP89	Knockout of gene	Aids in germination of rice under submerged conditions	Cas9	*Agrobacterium tumefaciens*	[72]

applications of CRISPR/Cas for managing abiotic stress and quality traits in crop plants (Table 2).

Drought

Ethylene plays an important role in regulating water and high-temperature stresses in plants. Several studies have shown that reducing the ethylene biosynthesis under drought stress improves the grain yield of maize as well or conversely the plant yield can be improved by reducing the sensitivity of plants to ethylene. ARGOS is known to negatively regulate the response of the ethylene under drought stress and overexpression of this gene has conferred drought stress tolerance in maize. CRISPR/Cas9 method has been used to edit the promoter region of the *ARGOS8* to increase the expression of this gene under different tissue and growth stages of the maize to create breeding lines [70]. The alteration of the expression of a single gene resulted in

the increase of maize grain yield under the drought stress in field experiments.

Drought stress in plants results in ROS accumulation because of oxidative damages. Plants require enzymes such as superoxide dismutase and catalase, which have high ROS scavenging activity [71]. Abscisic acid (ABA) plays an important role in acquiring drought tolerance mechanisms in plants. SAPK2, the primary mediator of the ABA signaling in the rice, was characterized using a loss of function created with CRISPR/Cas9 mutation in the third exon. SAPK2 is shown to regulate the expression of polyethylene glycol under salinity and drought, while its mutant *sapk2* exhibits the ABA insensitive phenotype [71]. This *sapk2* mutant could not scavenge ROS and was sensitive to drought stress providing evidence that SAPK2 plays an important role in drought stress in rice. SAPK2 regulates drought stress with reduction of water loss by closing the stomata, increasing the synthesis of the compatible solutes, inducing the expression of ROS scavenging hormones to reduce ROS damage, and upregulating the expression of stress regulating genes. OsEBP89 has also been found to involve in drought tolerance in rice by increasing the scavenging of ROS and accumulation of proline in the cells under drought stress [72]. The knockout of this gene with the help of CRISPR/Cas9 induces the expression of several genes that regulate the adverse effects in the plant.

Mitogen-activated protein kinases (MAPKs) play an important role as signaling molecules for drought stress. SlMAPK3 is a class of MAPKs and their knock out in tomato using CRISPR/Cas9 proved its involvement in drought tolerance [73]. The mutant *slmapk3* exhibited severe wilting symptoms, more cell membrane damage, lower accumulation of antioxidant enzymes, and higher hydrogen peroxide content. Moreover, the mutant reduces the expression of several drought-responsive genes in the plant, thus concluding that *SlMAPK3* increases drought tolerance in tomatoes by protecting the plant from oxidative damages. Non-expressor of pathogenesis-related gene 1 (*SLNPR1*) is involved in drought stress response in the tomato, and its knockout by the CRISPR/Cas9 resulted in reduced expression of several drought-related key genes [74]. The mutant exhibited reduced drought tolerance because of increased stomatal aperture, reduction in the synthesis of antioxidant enzymes, and higher electrolytes leakage [74].

Salinity, heavy metals, and flooding tolerance

Salinity is an issue impending the agricultural production over the natural highly saline soil or land having poor water management strategies. Osmotic stress is induced in plants because salinity results in the closure of the stomata, reduction in water uptake, and ultimately reducing the plants photosynthetic efficiency [46]. Rice is mainly grown in freshwater marshes or swamps, because of which it is highly susceptible to salt stress, suggesting the urgent need for targeting the breeding goal in this direction. The salinity tolerance in rice was increased by creating multiple small insertion and deletion mutations in the *OsRR22* gene using CRISPR/Cas9 without affecting the agronomic traits [46]. In the case of soybean, CRISPR/Cas9 and TALENS were used synergistically for the creation of double mutants having improved tolerance to salinity. It involved the processing of double-stranded RNA into small RNA, and these mutations were observed to be germline transmissible for breeding salt tolerant lines [75].

A significant portion of the agricultural land is contaminated with heavy metals such as Pb, Cs, As, etc., because of fertilizers, pesticides, municipal wastes, and contamination from the industries. Most of these heavy metals have entered the plant and human food chain because of uptake of these elements by plants from the soil when they are present at an elevated concentration leading to toxic symptoms in the plants as well. Toxic plants act as the primary source of the entry of these heavy metals into the human food cycle, causing several deadly diseases such as cancer, diarrhea, etc. [76]. Current breeders, geneticists, and physiologists are working hard to reduce plant contamination with such heavy metals and avoid their entry into the food chain. Rice gene *OsNramp5* plays an important role in transporting cadmium to the rice grain, where it enters humans, causing deadly diseases. This gene's knockout resulted in less cadmium accumulation in shoots and roots than the wild type under the high cadmium soil conditions [76]. Mutant plants didn't have any yield penalty and were highly safe for human diets. Similar targets, *OsLCT1* and *OsNramp5* in rice, were targeted using CRISPR/Cas9 for decreasing the cadmium accumulation, and the knockouts of both the genes showed a reduction in cadmium accumulation. The knockout of *OsLCT1* is more effective in low cadmium affected soils while the knockout of *OsNramp5* is highly effective in high cadmium soils [77]. *OsARM1* is involved in arsenic transportation in rice, and the knockout of this gene with the help of the CRISPR/Cas9 system suggested its role in arsenic uptake. The mutant plant showed decreased accumulation of arsenic in different parts of the rice plant and could be used as a source for development and identification arsenic tolerant lines [78]. Several other studies demonstrate the use of CRISPR/Cas for reducing heavy metal stresses in the plants (Table 2).

With the rise in global temperature, the incidence of floods is increasing at a rapid pace. This scenario is crucial for direct-seeded rice because flooding events in Asia coincide with rice germination time [72]. *OsEBP89* plays a vital role in submergence tolerance in rice, as knocking

out of this gene aids in proper germination under the submerged soil conditions. Further, the mutant plant exhibited an improved ability to scavenge ROS and higher proline accumulation to deal with stress conditions [72].

Herbicide resistance

Glyphosate is one of the most important and rapidly adopted herbicides for application in resistant crops such as maize, soybean, sugar beet, and chili pepper. The development of glyphosate-resistant crops requires alterations in the mechanism of some genes [79]. Enzyme 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS) is involved in the synthesis of aromatic compounds in the plants with the transfer of phosphoenolpyruvate (PEP) for triggering the reaction [79]. Glyphosate inhibits the action of the EPSPS enzyme by inhibiting the attachment of glyphosate to the PEP binding sites, ultimately blocking the synthesis of aromatic products and causing plant death [79]. Rice endogenous *EPSPS* gene was targeted with CRISPR/Cas9 to create site-specific gene insertions and replacement, which were fully transmitted to the next generation with plants fully resistant to the glyphosate [79]. CRISPR/Cas9 was used for creating a mutation in the promoter of the *EPSPS* gene in chili to express this gene under the action of glyphosate [80]. The resulting plants were moderately resistant to glyphosate, and further studies suggested that selecting a different promoter will aid in the development of completely resistant chili [80].

The gene *ACETOLACTATE SYNTHASE* (*ALS*) encodes the enzymes controlling branched-chain amino acid synthesis in plants. Chlorosulfuron is a broad-spectrum herbicide used for targeting the *ALS* in weeds and ultimately causing their programmed cell death [81]. Different crops have been modified for *ALS* to confer resistance to this herbicide by targeting several amino acids in the gene sequence [81]. Cytidine base editor was used to create point mutation in the *ALS* to develop edited tomato and potato having resistance to Chlorosulfuron [81]. This base editing helps in reducing the deleterious effects of transgenes by avoiding the random insertion of genes in the genome. The multiple point mutations in the *ALS* gene of rice were induced using a particle bombardment approach to create Chlorosulfuron resistant lines [82]. Similarly, the CRISPR/Cas9 system was utilized to create small insertions and deletions in soybean *ALS* for conferring resistance to the chlorsulfuron herbicide [83]. Several other herbicides inhibit the action of *ALS* genes, namely Imidixolinone, Tribenuron, Nicosulfuron, and Mesosulfuron. Geminivirus have also been employed for targeting sequence-specific nucleases to create point mutations in the *ALS* gene of potato to impart resistance against Imidixolinone herbicide [43]. Tribenuron is a broad-spectrum herbicide used for controlling broadleaf weeds, and its application in watermelon results in plants death as it interferes with the functioning of ALS protein [84]. CRISPR/Cas9 mediated conversion of Cytosine to Thymine in ALS protein resulted in herbicide resistance watermelon plants [84]. Several herbicides have been safely used on crop plants modified through CRISPR/Cas-based systems (Table 2).

Heat and cold stress

Rice is an important crop in Asia, especially in China, where they use hybrid rice production with a 10–25% yield increase. There are mainly two systems used for hybrid seed production, viz. three-line, and two-line systems. The three-line system requires cytoplasmic male sterile, maintainer, and restorer lines. However, the biggest issue with the constant exploitation of these three sources has resulted reducing genetic variations for making selections. CRISPR/Cas9 provides a valuable tool for creating a hybrid using the two-line hybrid mating system to create thermo- or photoperiod-insensitive genic male sterile lines for developing hybrid rice seed. Thermo-sensitive genetic male sterile line for use in the hybrid seed production was created using CRISPR/Cas9 with a single nucleotide change in the *TMS5* gene sequence [85]. This two-line system is comparatively advantageous over the three-line system in providing high grain yield, being less labor intensive, time saving, and utilizes simple breeding processes. Similarly, CRISPR based genome editing system was utilized to develop a two-line system in hybrid maize with the development of thermo-sensitive genetic male sterile lines [86]. CRISPR/Cas9 was used to create small insertions and deletions in the various regions of the genome. The resulting lines were verified for their heat tolerance abilities, as summarized in Table 2.

Parthenocarpy acts as a golden opportunity for fruit crops and vegetables to combat heat stress. Reproduction is highly susceptible to heat stress due to the adverse effect on the microsporogenesis process during fruit development, e.g., in tomatoes. Herein, parthenocarpy acts as an important breeding objective for maintaining the sustainable production of agriculture amid the high temperature and global warming pressure. *SlAGL6* gene responsible for parthenocarpic fruit setting was identified from tomato under heat stress [87]. CRISPR/Cas9 mediated gene knockout confirmed the parthenogenic phenotype with same fruit size, yield, quality attributes, and sexual reproduction capacity as under normal fruit setting.

Previously, genome editing was inefficient in targeting polyploid species like wheat because of the homologous chromosomes, thus reducing the target-specific changes. CRISPR/Cas9 was employed for editing the wheat protoplast by targeting the wheat ethylene-responsive factor 3 (*TaERF3*) and wheat dehydration responsive element

binding factor protein 2 (*TaDREB2*), which are known to regulate heat stress [88]. The effectiveness of this gene-editing was confirmed using restriction enzymes and sequencing assays, and it was noticed that there was no off-target editing. This study demonstrated that CRISPR/Cas9 could be efficiently used in editing wheat genes for maintaining stable performance under heat stress by silencing dehydration responsive elements.

The C repeat bind factor (CBF) is an essential regulator for the expression of cold-regulated genes (COR) in most crop and tree plants [89]. The CBF regulates the expression of the *COR* genes by targeting the cis-acting elements of these genes. Most of the CBF occurs in tandem repeat and in multiple copies over the genome. CRISPR/Cas9 mediated knockout for creating small insertions and deletions in *SlCBF1* gene provided evidence for regulating chilling injury in tomatoes [89]. The mutant plant had higher electrolyte leakage, lower proline content, and severe chilling symptoms. The mutant also exhibited a higher accumulation of indole acetic acid, further verifying that the *SlCBF1* gene regulates chilling stress in tomatoes [89]. Knockout of *OsANN3* gene using CRISPR/Cas9 in rice and showed that it plays an important role in tolerating the chilling stress, as mutants exhibited poor survival [90, 91].

Risk assessment and Biosafety

Genome editing (GE) techniques have started gaining much attention for crop improvement owing to their unique feature of being highly target-specific as well as time-saving compared to conventional genetic engineering techniques. In spite of being highly specific compared to genetic transformation, uncontrolled radiation, or chemically induced mutations, there are still ample chances that GE may unintentionally alter closely resembling gene sequences and interfere with their well-organized network of biochemical and physiological pathways. Thus like conventional genetic engineering techniques, GE and GE products also require a well-defined regulatory and biosafety system conducive for their expansion and contribution towards the welfare of mankind. Most of the countries with an existing regulatory framework for GMOs are yet to decide whether to place GE procedures or products under the same legislation or to have a separate framework for their risk assessment and biosafety. The conventional GMOs carry foreign DNA, which can cause irregularities at the genome level leading to the transmogrification of plant metabolites and that may be a matter of concern for food, animal feed, and the environment as a whole [106]. However, some of the GE technologies such as Transcription Activator-like Effector Nucleases (TALEN), Meganucleases (MN), and Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR) may not always involve the integration of foreign DNA into the target site. The two Site Directed Nucleases categorized as SDN1 and SDN2 are responsible for delivering small deletions/ insertions in the target genome, N3 while SDN3 incorporates larger chunks of DNA into the target genome [107]. Thus it is very clear that the changes based on SDN1 and SDN2 are very similar to the naturally occurring mutations and thus their traceability and detection are challenging [108]. In view of this, there are opinions that SDN1 and SDN2 based GE products may be allowed for commercial use after their field evaluation as these are quite similar to mutation breeding-based products, while SDN3 based processes and products have to go through similar or more stringent regulation as in case of conventional transgenics [108, 109]. It is not possible even at any level of stringency in the risk assessment procedure to determine every minute sort of threat to the non-targets and its environment due to insufficient understanding of intended and unintended ill effects on the target organism, environment, and their interactions. Sometimes, due to these reasons, the risk assessment procedures for GMOs have been times challenged [109]. So, it becomes important to strengthen the risk assessment methodologies for any type of GE organism keeping in view enormous scientific uncertainties due to the limited knowledge of safety data. Studies suggest that GE products based on any approach i.e. SDN1, SDN2, or SDN3 can cause varying levels of irregularities in the genome through off-target effects [110, 111].

The safety protocols of any GE product should be able to trace each and every unintended effect among the various unanticipated effects caused in the resulting crops due to alterations in the biochemical and physiological processes of the target organism. These impacts may not only be limited to the physiological processes but may have major concerns to the safety of consumers and the environment due to alterations in the metabolomics and proteomics profile of the target organism. Thus thorough evaluation of all such effects is required for their adverse effects on the interacting environment before placing the product and its process for commercial use. The evaluation should not be limited to the recognition error of gRNA but must have complete considerations to the risk arising from epigenomic and genomic irregularities in the GE product. The prediction of target sites and the issue of off-target effects is being currently addressed through numerous *in silico* tools, however, there are many loopholes in their reliability as these may sometimes fail to detect the irregularities at physiological or biochemical or genomic, or proteomic levels [112]. There are suggestions to address these issues through genome sequencing, however, the major limitation in this technique is to differentiate between the natural variations and

Table 3 Status of regulations being adopted by various countries across the globe for genome-edited products and processes

S#	Category	Countries
1	Non-regulated	United States of America
2	Regulated	European Union, Canada, India, Malaysia, New Zealand, South Africa, Thailand, Mexico
3	Case-by-case, if no foreign DNA then not regulated	Argentina, Chile, Brazil, Colombia, Paraguay, Uruguay, Bangladesh, Nigeria, Kenya
4	Discussion ongoing	Phillippines, Indonesia, United Kingdom, Norway
5	If no foreign DNA then not regulated as GMO	Japan, Australia, Israel
6	No information on regulatory and biosafety of GE crops/ organisms/ products	Burma, China, African countries except Kenya and Nigeria. Russia and other European nations except those in EU, UK, and Norway. Mediterranean nations. Latin American nations- Bolivia, Cuba, Ecuador, French Guyana, Guyana, Peru, Venezuela, and other nations not listed in the above categories

Modified from Schmidt *et al.* 2020 [118]

unintended effects. For the first generations GMOs the information is sought for both intended and unintended effects under the European Union Directive of 2001 and Regulation # 1829/2003, however, the molecular data under these situations is restricted to the insert and flanking regions of the insertion site. These Directives may not be useful for the GE products due to specific site restrictions. Thus a holistic 'omics' approach to visualize or trace the changes in the whole genome, epigenome, transcriptome, proteome, and metabolome of the GE organisms with the help of robust bioinformatics tools is required to develop the risk assessment package for the GE products and processes [113]. Like GMOs, the regulation, release, and trade of GE technologies also fall under the decree of the Cartagena Protocol on Biosafety. The regulation of GMOs, as well as the GE products and processes, are not uniform across the globe with some countries having stringent policies on the table for their risk assessment and regulation, while others are still underway to frame such policies and protocols. [114–116]. The major bottleneck in framing the regulatory policies for GE products and processes is also because of difficulties in differentiating between naturally occurring genetic variations and SDN1 or SDN2 based products. Considering this the United States Department of Agriculture (USDA) in 2018 declared that the biosafety and commercialization of the products belonging to these categories may escape the strict regulatory guidelines because of their high similarity to some conventionally bred products [115, 116]. European Union on the other hand has strictly considered the regulation of GE-based products like first-generation GMOs and declared these to pass through strict procedures and protocols [117]. The GE product and process regulation guidelines adopted across the globe can be divided into six major categories as listed in Table 3 with the majority of countries still in process of making firm decisions on these issues. We hope that scientific evidences coming through advanced research and more discussion and deliberations will help in framing viable risk assessment regulations for GE products and processes.

Conclusions

Cultivated crops have been facing various biotic and abiotic stresses since the inception of agriculture; however, under the current scenario of meeting the food requirements of geometrically increasing population numbers, managing these stresses to avoid crop losses becomes necessary. The conventional strategies played a crucial role in developing crops that can withstand various biotic and abiotic stresses; however, these plant breeding techniques are complicated, time-consuming, and labor-intensive. These limitations can be coped up with recent cutting edge technologies like RNAi [119] or CRISPR/Cas-based systems, which offer higher efficiency and target specificity for biotic and abiotic stress management in crop plants. CRISPR-based tools have been widely employed in the plant system to understand the gene functions and consequently use this knowledge for crop genetic improvements. The CRISPR/Cas based genome plant genome engineering has been used to create single or multiple mutations at desired loci to either eliminate or integrate undesirable and desirable insertions for beneficial traits in plants, respectively. The most significant advantage of this tool is that it can be used for multiplex genome editing targeting multiple genes simultaneously, which is nearly impossible with conventional techniques. Within a few years of its introduction in agriculture, CRISPR/Cas system has been used to address various biotic and abiotic stresses in plant systems. This system has been used to understand the plant-pathogen interactions at the molecular level, which has helped to understand the plant defense system and enhance plant resistance against pathogens. So far, more than 20 crop species have been subjected to CRISPR/Cas gene editing in context to biotic and abiotic stress management and increase in yields. These include important staple food crops of the world such as rice, wheat, maize, and potato as well other crops like sorghum, tomato, apple, banana, soybean tobacco, cotton, etc. With the advancement in science, genome sequencing tools have become affordable, and this will boost the CRISPR/Cas9 research even in

under-utilized crops or other minor crops once their genome is sequenced. Thus CRISPR/ Cas9 based genome editing systems have a broad scope for genetic engineering of next generation future crops; however, despite these many advantages, there are some challenges related to this technology such as off-target effects, regulatory issues, and public acceptability. It is thus expected that intensive worldwide research on the CRISPR/Cas system in plants will indeed address its challenges and contribute to the durable resistance against biotic and abiotic stresses in plants.

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1007/s11033-022-07741-2.

Author contributions JS and KSS collaborate, outline the study, and invite the members; JS, DS, GSB, KSS, SHW, RK, AK, and SS write and edit the manuscript. All authors contributed equally to the work.

Funding The authors declare that no funds, grants, or other support were received during the preparation of this manuscript.

Data Availability There is no data pertaining to this study.

Declarations

Competing Interests The authors have no relevant financial or non-financial interests to disclose.

Ethics approval This is a review article, thus no ethical approval is applicable.

Consent to participate Not applicable.

Consent to Publish Not applicable.

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