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Mechanistic insights of CRISPR/Cas-mediated genome editing towards enhancing abiotic stress tolerance in plants

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Abstract

Abiotic stresses such as temperature (high/low), drought, salinity, and others make the environment hostile to plants. Abiotic stressors adversely affect plant growth and development; and thereby makes a direct impact on overall plant productivity. Plants confront stress by developing an internal defense system orchestrated by compatible solutes, reactive oxygen species scavengers and phytohormones. However, routine exposure to unpredictable environmental stressors makes it essential to equip plants with a system that contributes to sustainable agricultural productivity, besides imparting multi-stress tolerance. The sustainable approach against abiotic stress is accomplished through breeding of tolerant cultivars. Though eco-friendly, tedious screening and crossing protocol limits its usage to overcome stress and in attaining the goal of global food security. Advancement on the technological front has enabled adoption of genomic engineering approaches to perform site-specific modification in the plant genome for improving adaptability, increasing the yield and in attributing resilience against different stressors. Of the different genome editing approaches, CRISPR/Cas has revolutionized biological research with wider applicability to crop plants. CRISPR/Cas emerged as a versatile tool in editing genomes for desired traits in highly accurate and precise manner. The present study summarizes advancement of the CRISPR/Cas genome editing tool in its adoption to manipulate plant genomes for novel traits towards developing high-yielding and climate-resilient crop varieties.

1 | INTRODUCTION

Plants, represented as complex organisms, are exposed to a variety of factors (physical or chemical) that infringe strong impact on plant productivity (hindering their maximum performance) and even threaten their survival (Shao et al., 2015; Suzuki et al., 2014; Z. Zhu, Piao, et al., 2016). Of the different factors, drought, salinity, temperature (high or low), and others such as ultraviolet (UV) radiation, heavy metals, etc are prominent and collectively referred to as abiotic stresses (He et al., 2018; Mahajan & Tuteja, 2005; Sharma et al., 2020; Zafar et al., 2020). Any

exacerbation in the frequency of abiotic stresses affects plant from germination to maturity stage (Waqas et al., 2019). Abiotic stresses (acting individually or in combination) emerged as a potent threat that poses strong challenges to the survival of plants. Survival of plants under such adverse conditions relies on stress adaptive changes (both structural and metabolic) for integration into endogenous developmental program towards restoring cellular homeostasis (Golldack et al., 2014). Though abiotic stresses have shaped and continue to shape the evolution of plants, impairment of the physiological, biochemical and molecular processes proceeds with imposition of serious consequences on the growth and development of plants and overall reduction in their productivity (Boscaiu & Fita, 2020; Khan et al., 2015).

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Environmental stress—a major challenge of 21st century—presents an unpredictable constraint that increases the difficulties in securing adequate food supply for growing human population (Nadeem et al., 2018; Nadeem, Li, Yahya, Sher, et al., 2019; Shabala, 2013; Xu et al., 2014). Under such circumstances, it becomes imperative to have crop varieties that display better adaptability for growth under varied environmental conditions. Though, conventional breeding approach has succeeded to a large extent in achieving the goal of increased production, it encounters problem of losing the fitness and genetic diversity. Besides being a time-consuming practice, its dependence on natural allelic variations makes it inefficient for obtaining a desired trait towards achieving sustainability in production (T. Wang, Zhang, & Zhu, 2019). To this, novel methods are explored that can overcome the limitations of conventional breeding approaches in recognizing the factors and removing the roadblock that causes persistent loss in crops production or editing crop plants for improving the desired traits (Chen, Wang, et al., 2019). One such approach is editing plant genomes precisely and in an accurate manner towards achieving the targeted goal of fulfilling the demand of growing population. Herein, we confined our study to food security and abiotic stress, with special emphasis on the recent advances in CRISPR/Cas-mediated editing of crop plants to overcome the phenomenal dilemma of different abiotic stresses.

2 | ABIOTIC STRESS AND INTRINSIC SURVIVAL STRATEGIES

Abiotic stresses arise as a gradual process in the global climate change (Bray, 1997; Dai, 2013; Nadeem et al., 2018; Nadeem, Li, Yahya, Wang, et al., 2019). Abiotic stresses progress with reduction in the germination, decreased photosynthetic activity and carbon assimilation, repressed flowering and pollen sterility, and limited crop productivity (Croser et al., 2003, Samarah et al., 2009, Mondal et al., 2011, Zlatev & Lidon, 2012, Chowdhury et al., 2016, Sehgal et al., 2018). Affecting different aspects of the plant growth, the stress poses a serious threat to the survival of plants and accounts for huge loss in the crop productivity. The effect of abiotic stresses is often correlated with the soil water level as its availability is considered important for plant survival. Decrease in the soil water level causes reduction in cultivable capacity of the soil that proceeds with change in land use pattern from fertile to marginal (Bhat, Kumar, et al., 2020). Water scarcity and rise in temperature (perceived as outcome of the climate change) adversely affect biological activities such as turgor, chlorophyll content, rate of photosynthesis, carbon assimilation, nutrient acquisition and cellular metabolism, gaseous exchange at leaf surface, and so on that together causes a reduction in the crop yield worldwide (Huang & Gao, 1999; Hussain et al., 2018; Nadeem, Li, Yahya, Wang, et al., 2019; Shao et al., 2008). Water stress encompassing both drought and salinity has shown predominance as unpredictable constraint in the salinization of major proportion of arable land around the globe (Ali et al., 2017; Ma et al., 2020). In the past decade, crop yield has shown a significant decline particularly for crops like rice,

wheat, corn; thereby severely impacting global food production (Boyer et al., 2013; Rosenzweig et al., 2014). Towards understanding the underlying mechanism, it was found that most of the abiotic stresses are accompanied by oxidative stress that exerts serious implications on macromolecules that ultimately progress to cell death via cell membrane damage (Demidchik, 2015; Gill & Tuteja, 2010).

In agriculture systems, abiotic stresses constraint plants in their normal cellular functioning and other physiological traits. The intensity and severity of the abiotic stress, such as water deficiency that hinders growth and development of the plants, constitutes a major constraint to global agricultural productivity (Nadeem, Li, Yahya, Sher, et al., 2019). The foremost strategy adopted by the plants to grow in water deficient environments is adoption of the drought escape (DE) mechanism that involves rapid growth and reduced life span to match the period of soil water availability (Farooq et al., 2014; Siddique et al., 1993). As part of the survival strategies, osmotic homeostasis critical for normal cellular function is maintained by accumulation of the osmoregulatory compounds such as proline and mannitol (Gujjar et al., 2018; Krasensky & Jonak, 2012). Besides acting as a signaling molecule in regulating mitochondrial function, activation of the stress responsive genes by proline helps in regulating cell proliferation. Additionally, accumulation of proline proceeds with decrease in the lipid oxidation via, scavenging of free radicals; thereby maintains the structural integrity of the membrane (Ramanjulu & Bartels, 2002; Shinde et al., 2016). Mannitol assists in stabilizing cellular reductants such as glutathione (GSH) and ferredoxin (Fd) and helps in scavenging hydroxyl ($\cdot\text{OH}$) radicals (Bhauso et al., 2014; Shen et al., 1997). Besides regulating the physiological function, maintenance of cellular turgor and stomatal movement by compatible solutes causes improvement in the photosynthesis and overall plant growth.

The oxidative stress enhances the production of reactive oxygen species (ROS), such as superoxide ($\text{O}_2^{\cdot-}$), hydroxyl ($\cdot\text{OH}$) radical, and hydrogen peroxide (H_2O_2), which are capable of damaging biological macromolecules such as proteins, DNA, and lipids (Demidchik, 2015; Gill & Tuteja, 2010). Therefore, controlling the production of ROS via enhancement in ROS scavenging by enzymatic and non-enzymatic components constitutes an important aspect for maintaining cellular homeostasis and in enhancing multi-stress tolerance in plants (Greco et al., 2012; Turk et al., 2014). The goal is achieved by genes encoding functional enzymes such as glutathione reductase (GR), glutathione peroxidase (GPX), ascorbate peroxidase (APX; generates NADP^+ and as such converts H_2O_2 to H_2O), dehydroascorbate reductase (DHAR; assists in providing substrate for reaction), catalase (CAT; detoxifying H_2O_2 to H_2O), superoxide dismutase (SOD; detoxifying $\text{O}_2^{\cdot-}$) along with other non-enzymatic cellular reductants such as glutathione, ascorbic acid, etc., which are capable of scavenging the ROS (Gill & Tuteja, 2010; Parmar et al., 2017). Plants primed for abiotic stress were observed to have improved functionalities associated with both enzymatic and non-enzymatic systems. Additionally, phytohormones such as auxin, gibberellins, ethylene enhance stress tolerance by assisting in regulating different steps of growth and development in plants (Bielach et al., 2017; Ullah et al., 2018; Weyers & Paterson, 2001).

3 | MECHANISTIC INSIGHTS OF CRISPR/CAS-MEDIATED GENOME EDITING

Rudd Jansen and colleagues coined the term “CRISPR” for multiple short (21–37 bp) repeat sequences (Jansen et al., 2002). CRISPR (Clustered Regularly Interspaced Short Palindromic Repeats) family of repetitive sequences (interspersed non-repetitive sequences in between short repetitive sequence repeats) is flanked by a leader sequence of 300–500 bp. Francisco Mojica reported the presence of conserved motifs in *Streptococcus thermophilus* associated with protospacer and designated them as protospacer adjacent motifs (PAM) (Mojica et al., 2009). The PAM sequences (2–5 nucleotides) were later found assisting in recognition and cleavage of DNA upstream of the PAM in a site-specific manner by Cas (CRISPR associated sequences) nuclease (Gleditsch et al., 2019). The most used Cas for genomic editing application is Cas9 from *Streptococcus pyogenes* (SpCas9) (Le Rhun et al., 2019). It requires canonical PAM sequence 5'NGG3' (where “N” is A, T, G, or C) for site-specific cleavage at the target site. As pre-requisite for cleavage by CRISPR/Cas, SpCas9 has been engineered to enhance recognition of a broad range of PAM sequences (NGG, GAA, and GAT) juxtaposed at 3' position of the target sequence (Wada et al., 2020). Addition to recombinant SpCas9, Cas variants reported from diverse biological groups engineered for recognizing broader PAM sequences improved specificity in recognizing otherwise inaccessible (sequence that escape recognition by SpCas9) target sequences in the genome (Hu et al., 2018) (Table 1).

Transcription of the CRISPR array led to the formation of mature CRISPR RNA (crRNA; characterized by PAM motif localization in front of the protospacers) via its precursor CRISPR RNA (pre crRNA) (Hille

et al., 2018). Maturation of crRNA requires a transacting crRNA (tracrRNA) (Deltcheva et al., 2011). The maturation process often leads to formation of a chimera of crRNA and tracrRNA; referred to as short guide RNA (sgRNA; nowadays generated as fusion construct between crRNA and tracrRNA) that along with Cas (sgRNA-Cas complex) scan the genome for PAM sequences so as to make site-specific cleavages (double stranded break in DNA) in the genome (Bhat, Bhat, et al., 2020; Lone et al., 2018; Rahman et al., 2019; Zaychikova et al., 2020). Recognition of the target DNA in the cleavage process is accomplished by crRNP complex and its cleavage at defined sites is facilitated by HNH- and RuvC-like nuclease domains (Lone et al., 2018).

The serendipity of CRISPR/Cas genome editing system depends largely on recognition of the PAM sequences at target sites in the genome (Adli, 2018; Wu et al., 2014). The site-specific recognition is followed by a double-strand break (DSB) via, sequence-specific nucleases (Cas variant) upstream of the PAM sequences at the targeted genome region. It is followed by repair of the DSB using homologous direct repair (HDR, error free) or non-homologous end-joining (NHEJ, error prone) methods (Manghwar et al., 2019). The HDR involves replacement of the cleaved section with user-defined synthetic construct, while as the latter one possibly induces one or two substitutions or small insertions-deletions (INDELS) at the site of cleavage (Figure 1). The selection between HDR and NHEJ pathways to repair the DSB largely depends on DNA end break and cell cycle phase (Yang et al., 2020). Although, HDR utilizes allelic sequence homology of the template in repairing the DSB, a bias towards NHEJ (involving direct ligation of free ends) observed among somatic cells helps in ensuring genomic integrity among complex genomes (Tang

TABLE 1 Cas variants and their specificity for diverse protospacer adjacent motifs (PAM) sequences

S. no.	Cas class	PAM sequence	Host	Reference(s)
1.	SpCas9-NG	5'-NAC, NTG, NTT, and NCG apart from NG-3' PAM	<i>Streptococcus pyogenes</i>	Ren et al. (2019)
2.	Cas14	Thymine-rich PAM sequences	Uncultivated archaea	Harrington et al. (2018)
3.	Cas13	Non-G nucleotide at the 3' protospacer flanking site (PFS)	Multiple orthologs; <i>Leptotrichia shaii</i>	Garcia-Doval and Jinek (2017)
4.	Cas12a (Cpf1)	Thymine-rich PAM sequences (TTTN or TTN)	<i>Acidaminococcus</i> sp. BV3L6; <i>Prevotella</i> and <i>Francisella</i> sp.	Jeon et al. (2018), Bin Moon et al. (2018)
5.	xCas9	NG, GAA, and GAT	<i>S. pyogenes</i>	J. Wang, Meng, et al. (2018)
6.	AacC2c1	T-rich PAM	<i>Alicyclobacillus acidoterrestris</i>	Liu et al. (2017)
7.	CjCas9	NNNNACAC and NNNRYAC	<i>Campylobacter jejuni</i>	Kim et al. (2017)
8.	Cpf1 (AsCpf1)	5'-TTTN-3'	<i>Acidaminococcus</i> sp.	Yamano et al. (2016)
9.	FnCas9	5'-NGG-3'	<i>Francisella novicida</i>	Hirano et al. (2016)
10.	Nme Cas9	5'-NNNNGATT-3'	<i>Neisseria meningitidis</i>	Lee et al. (2016)
11.	St1Cas9	NNAGAAW	<i>Streptococcus thermophilus</i>	Kleinstiver et al. (2015)
12.	SaCas9	5'-NNGRRT-3'	<i>Staphylococcus aureus</i>	Nishimasu et al. (2015)
13.	St3Cas9	5'-NGGNG-3'	<i>S. thermophilus</i>	Cong et al. (2013)
14.	SpCas9	5'-NGG-3'	<i>S. pyogenes</i>	Jinek et al. (2012)

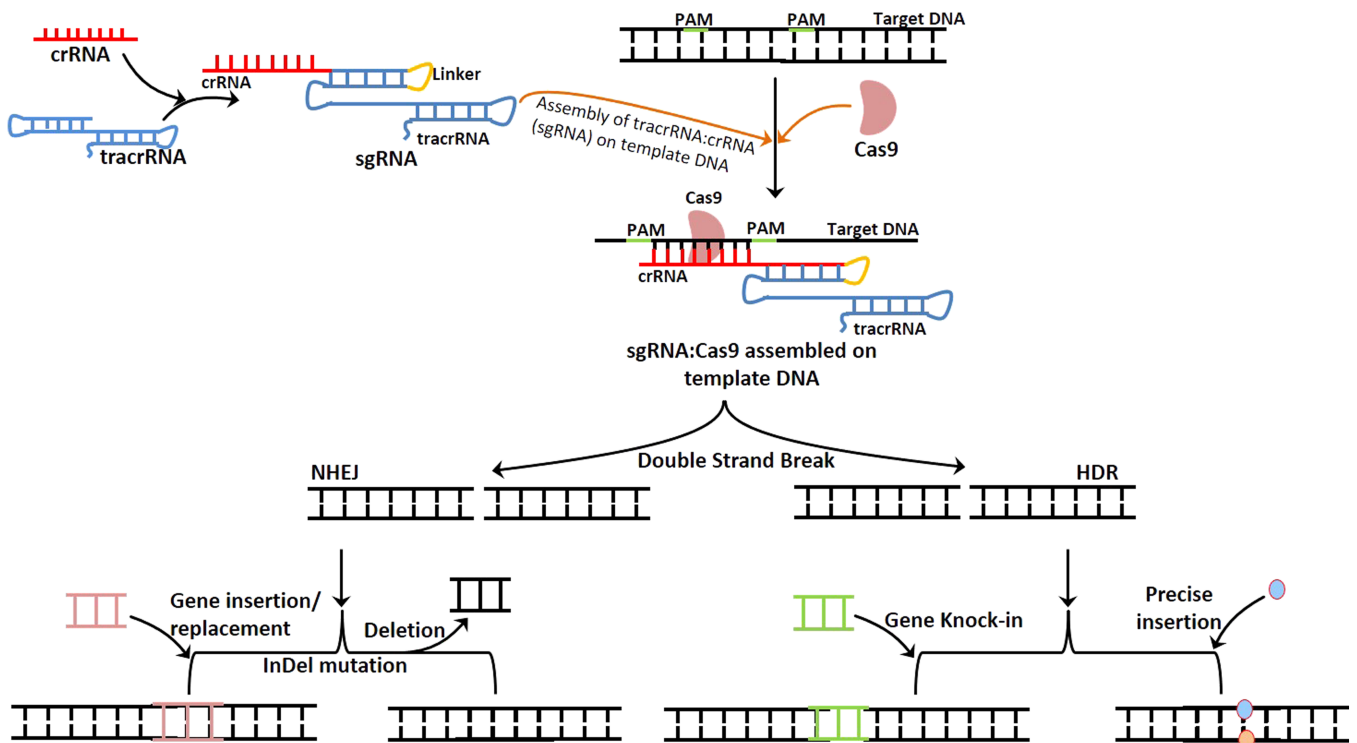


FIGURE 1 Mechanistic insights of CRISPR/Cas editing in plants. The functioning of CRISPR/Cas module begins with the assembly of sgRNA-Cas9 on the target DNA followed by editing at the target site using PAM sequences and then repair of the double strand break (DSB) by homologous direct repair (HDR) or non-homologous end joining (NHEJ) repair system

et al., 2019). In the editing procedural program, the spacer sequences in guide RNA can be altered to guide Cas to edit different targets within the genome (Jiang et al., 2013). A thorough understanding of the repair system is essential to achieve precision over target gene modification towards generating modified plant genomes efficiently.

4 | CRISPR/CAS-MEDIATED PLANT GENOME EDITING

The substantial development of CRISPR/Cas genome editing tool with integration of the advanced approaches has lead biologists to edit genomes with greater efficiency and in a precise manner never imagined before (Cong et al., 2013; Doudna & Charpentier, 2014). The CRISPR/Cas editing module has opened new avenues of editing plant genomes towards improvement in terms of desired traits and in developing varieties resistant to different stresses (Baruah et al., 2017; Chandrasekaran et al., 2016; Chikkaputtaiah et al., 2017; Hassan et al., 2020). The CRISPR/Cas technique for trait improvement has successfully been used to edit genomes of crop plants such as maize, rice, tomato, soybean, sorghum, flax, camelina, cotton, rapeseed, lettuce, potato, cucumber watermelon, grapefruit, apple, and oranges (Lin et al., 2020; Ricroch et al., 2017; Zhang et al., 2016). It is reported that CRISPR/Cas editing module can prolifically be used to target the complex quantitative genes linked directly or indirectly to abiotic stress factors (Mushtaq et al., 2018). CRISPR/Cas-based editing of genes viz., *phytoene desaturase* (*OsPDS*), *mitogen-activated protein*

kinase (*OsMPK2*), and *betaine aldehyde dehydrogenase* (*OsBADH2*) in rice was found conferring abiotic stress tolerance (Shan et al., 2013; Shan et al., 2015). The editing rates observed were almost 9 and 7% for *OsPDS* and *OsBADH2*, respectively. In rice, *OsMPK5* (negative regulator of stresses) was selected for targeted mutagenesis followed by testing in protoplasts using a set of three gRNAs. The rice genome has been targeted for several genes like *stromal processing peptidase* (*SPP*), *young seedling albino* (*YSA*), and *outermost cell-specific gene5* (*ROC5*) for improved tolerance to abiotic stresses (Feng et al., 2013). Other genes edited and knocked by CRISPR/Cas include *OsPDS* (*phytoene desaturase*) gene and promoter regions regulating protoplast development (Jiang et al., 2013; Shan et al., 2013; Shan et al., 2015). Abiotic stress tolerance in plants was increased by editing the genome for ethylene responsible factor (ERF, a transcriptional factor) of AP2/ERF superfamily (Debbarma et al., 2019). In rice, CRISPR/Cas editing of stress/ABA-activated protein kinase 2 (*SAPK2*) was found mediating stress tolerance (Lou et al., 2017). The employability of CRISPR/Cas in editing plant genomes accurately and precisely has broadened its applicability as a promising strategy in agriculture.

5 | CRISPR/CAS EDITING FOR ABIOTIC STRESS TOLERANCE

Increased dwelling and urbanization has caused a reduction in the agricultural land that adds on to the dire needs of having a proper management policy in executing the schemes set for reducing its

TABLE 2 Genes targeted by CRISPR/Cas system for imparting tolerance against abiotic stress

Crop	Target gene	Delivery mode	Function	Reference(s)
High/Low temperature				
Rice (<i>Oryza sativa</i>)	<i>OsNAC006</i>	<i>Agrobacterium tumefaciens</i>	As transcription factor	Wang et al. (2020)
	<i>OsMYB30</i>	<i>A. tumefaciens</i>	Cold tolerance	Zeng et al. (2020)
	<i>Ann3</i>	<i>A. tumefaciens</i>	Cold tolerance	Romero and Gatica-Arias (2019)
Tomato (<i>Solanum lycopersicum</i> L.)	<i>BZR1</i>	<i>A. tumefaciens</i>	Brassinosteroid regulator	Yin et al. (2018)
	<i>CBF1</i>	<i>A. tumefaciens</i>	Transcription activator	Li et al. (2018)
	<i>ANN3</i>	<i>A. tumefaciens</i>	Stress response	Shen et al. (2017)
<i>Arabidopsis thaliana</i>	<i>CBFs</i>	<i>A. tumefaciens</i>	As transcription factor	Jia et al. (2016)
Drought stress				
<i>Brassica napus</i>	<i>BnaA6.RGA</i> (DELLA Protein)	<i>A. tumefaciens</i>	As transcription factor	Wu et al. (2020)
Rice (<i>O. sativa</i>)	<i>SRL1, SRL2</i>	<i>A. tumefaciens</i>	Rolling of leaf	Liao et al. (2019)
	<i>NAC14</i>	<i>A. tumefaciens</i>	As transcription factor	Shim et al. (2018)
	<i>SAPK2</i>	<i>A. tumefaciens</i>	ABA signaling	Lou et al. (2017)
	<i>DERF1, PMS3, EPSPS, MSH1, MYB5, SPP</i>	<i>A. tumefaciens</i>	Amino acid synthesis	Zhang et al. (2014)
Tomato (<i>S. lycopersicum</i> L.)	<i>NPR1</i>	<i>A. tumefaciens</i>	Drought tolerance	Li et al. (2019)
	<i>MAPK3</i>	<i>A. tumefaciens</i>	Growth and development	Wang et al. (2017)
	<i>NPR1</i>	<i>A. tumefaciens</i>	Drought tolerance	Wang et al. (2015)
Wheat (<i>Triticum aestivum</i>)	<i>DREB2, DREB3, ERF3</i>	PEG-mediated transformation	<i>Dehydration responsive element binding protein</i>	Kim et al. (2018)
Maize (<i>Zea mays</i>)	<i>ARGOS8</i>	Particle bombardment	Ethylene responsive gene family regulator	Shi et al. (2017)
<i>A. thaliana</i>	<i>AREB1</i>	<i>A. tumefaciens</i>	ABA signaling	Roca-Paixão et al. (2019)
	<i>Vacuolar H⁺-pyrophosphatase (AVP1)</i>	<i>A. tumefaciens</i>	As transcription factor	Park et al. (2017)
	<i>OST2</i>	<i>A. tumefaciens</i>	Stomatal movement	Osakabe et al. (2016)
	<i>miR169a</i>	<i>A. tumefaciens</i>	Negative factor of drought tolerance	Zhao et al. (2016)
Salinity stress				
Rice (<i>O. sativa</i>)	<i>GTγ-2</i>	<i>A. tumefaciens</i>	As transcription factor	Liu et al. (2020)
	<i>PIL14</i>	<i>A. tumefaciens</i>	As transcription factor	Mo et al. (2020)
	<i>PQT3</i>	<i>A. tumefaciens</i>	Ubiquitin ligase	Alfatih et al. (2020)
	<i>BGE3</i>	<i>A. tumefaciens</i>	As cytokinin transport	Yin et al. (2020)
	<i>DST</i>	<i>A. tumefaciens</i>	Zinc finger transcription factor	Santosh Kumar et al. (2020)
	<i>FLN2</i>	<i>A. tumefaciens</i>	As sucrose metabolism	G. Chen, Hu, et al. (2019)
	<i>RR9, RR10</i>	<i>A. tumefaciens</i>	Signaling of cytokinin	W. C. Wang, Lin, et al. (2019)
	<i>DOF15</i>	<i>A. tumefaciens</i>	As transcription factor	Qin et al. (2019)
	<i>SPL10</i>	<i>A. tumefaciens</i>	As transcription factor	Lan et al. (2019)
	<i>NCA1a, NCA1b</i>	<i>A. tumefaciens</i>	Chaperones Regulate activity of catalase	Liu et al. (2019)
	<i>RR22</i>	<i>A. tumefaciens</i>	As transcription factor	Farhat et al. (2019), A. Zhang, Liu, et al. (2019)
	<i>NAC041</i>	<i>A. tumefaciens</i>	Gene encoding transcription factor	Bo et al. (2019)
	<i>OTS1</i>	<i>A. tumefaciens</i>	Salt stress response regulator	C. Zhang, Srivastava, and Sadanandom (2019)

(Continues)

TABLE 2 (Continued)

Crop	Target gene	Delivery mode	Function	Reference(s)
	SAPK1, SAPK2	<i>A. tumefaciens</i>	ABA pathway regulator	Lou et al. (2018)
	BBS1	<i>A. tumefaciens</i>	Chaperones-mediate signaling	Zeng et al. (2018)
	MIR528	<i>A. tumefaciens</i>	Salt stress response regulator	Zhou et al. (2017)
	SAPK2	<i>A. tumefaciens</i>	ABA signaling	Lou et al. (2017)
	RAV2	<i>A. tumefaciens</i>	As transcription factor	Duan et al. (2016)
Maize (<i>Z. mays</i>)	HKT1	<i>A. tumefaciens</i>	High affinity potassium transporter	M. Zhang, Cao, et al. (2018)
Tomato (<i>S. Lycopersicum</i>)	CLV3	<i>A. tumefaciens</i>	Regulates shoot and Floral meristem development	Li et al. (2018), Van Eck et al. (2019)
	SP5G, SP	<i>A. tumefaciens</i>	Regulates daylength sensitivity	Li et al. (2018), S. Zhang, Jiao, et al. (2018)
	GGP1	<i>A. tumefaciens</i>	Vitamin C synthesis	Li et al. (2018)
	WUS	<i>A. tumefaciens</i>	Represses and activates gene transcription in shoot apical meristem	Li et al. (2018)
Multiple stress tolerance				
Maize (<i>Z. mays</i>)	PSY1	<i>A. tumefaciens</i>	Regulator of phytoene synthase	J. Zhu, Song, et al. (2016)
Tomato (<i>S. lycopersicum</i> L.)	PDS	<i>A. tumefaciens</i>	Carotenoid biosynthesis	Woo et al. (2015)
Rice (<i>O. sativa</i>)	AOX1a, AOX1b, AOX1c, BEL	<i>A. tumefaciens</i>	Breeding stress marker	Xu et al. (2015)
	PDS, MPK2, BADH2	Particle bombardment	Pigment synthesis	Shan et al. (2013)
	MPK5	<i>A. tumefaciens</i>	Defense response regulator	Xie and Yang (2013)
Wheat (<i>T. aestivum</i>)	INOX, PDS	<i>A. tumefaciens</i>	Inositol oxygenase, Phytoene desaturase	Upadhyay et al. (2013)
Herbicide resistance				
Rice (<i>O. sativa</i>)	ALS, C287T	<i>A. tumefaciens</i>	Acetolactate synthase, Imazamox (IMZ) resistance	Shimatani et al. (2017); Sauer et al. (2016)
	EPSPS	Particle bombardment	Synthesis of amino acids (aromatic)	Li et al. (2016)
	ALS	<i>A. tumefaciens</i>	Acetolactate synthase	Endo et al. (2016)
Wheat (<i>T. aestivum</i>)	EPSPS	Particle bombardment	Synthesis of amino acids (aromatic)	Arndell et al. (2019)
Tomato (<i>S. lycopersicum</i>)	ALS	<i>A. tumefaciens</i>	Acetolactate synthase	Veillet et al. (2019)
Maize (<i>Z. mays</i>)	ALS	Biolistic transformation	Acetoacetate synthase	Yadava et al. (2017)
	MS26, MS45	Biolistic transformation	Male fertility gene	Svitashev et al. (2015)
	LIG1	Biolistic transformation	Liguleless 1	Svitashev et al. (2015)
Soyabean (<i>Glycine max</i>)	ALS1	Particle bombardment	Acetolactate synthase	Li et al. (2015)

negative impact on agricultural practices. The current trajectory for yield of crop plants does not suffice to meet the need to feed the growing population (Ray et al., 2013). From the current indices, it is believed that the current productivity of crops such as rice, wheat, barley needs to be increased by >50% in order to have adequate food for humans towards achieving United Nations “zero hunger goal” that is projected for 2030 (Wheeler & von Braun, 2013). To circumvent the climatic

change and extreme weather conditions that limits crop productivity, it is imperative to develop varieties of crops that can grow under conditions of water scarcity and have traits for tolerance to different stresses towards improving the yield (Khanal & Mishra, 2017; Khoury et al., 2014). The following sections cover the role of CRISPR/Cas system in engineering crop plants for tolerance to drought, salt, temperature (high and cold), and resistance to herbicides.

5.1 | Engineered plants for temperature tolerance

Gradual change in temperature has profound effect on the adaptation and survival of crop plants (Bita & Gerats, 2013). A temperature rise of 5–15°C above the normal range indicates a well-defined heat stress, under which crop plants begin to show effects on growth and development (Fragkostefanakis et al., 2015). In response to heat stress of different strength and duration, diverse mechanisms involving alteration in the expression of genes encoding scavenger proteins, heat shock proteins, enzymes for synthesis of ROS, and enzymes that trigger accumulation of metabolites important for normal cellular functioning are initiated as part of the plant defense (Fragkostefanakis et al., 2015; Shah et al., 2013). The CRISPR/Cas9 genome editing module has evolved as the most successful tool in modulating genes essential for the development of thermotolerant crops (Biswal et al., 2019; Nguyen et al., 2018) (Table 2). Tomato (*Solanum lycopersicum* L.) is considered as an ideal model for testing editing through the CRISPR/Cas9 system owing to its ability to undergo efficient transformation for achieving quality improvements (Pan et al., 2016). CRISPR/Cas-mediated production of the deletion mutants of *HSA1* (*heat-stress sensitive albino 1*) exhibits higher sensitivity to heat than wild tomato plants (Qiu et al., 2018). Though 20–32°C is considered optimum temperature for observing peak photosynthesis in tomato plants, subsequent increase beyond this point causes a decrease of 5–60% (depending on stage of plant growth) in the rate of photosynthesis (Ruiz-Vera et al., 2015). Exposure of tomato plants to increased temperature in the range of 43–45°C led to significant reduction (< 20%) in the viability of pollens (Frank et al., 2009). The heat-tolerant tomato plants developed by CRISPR/Cas editing of the *slagamous-like 6* (*SIAGL6*) gene result in the generation of parthenocarpic fruits (Klap et al., 2017). From the above study, *Slagl6* was found a potential gene for employment in the study of facultative parthenocarpy. Mutation of the *thermosensitive genic malesterile 5* (*TMS5*) gene by CRISPR/Cas editing module results in the development of thermosensitive male sterile maize plants (Li et al., 2017). CRISPR-*bzr1*-mutant and *BZR1*-overexpressing tomato lines verified the involvement of *BZR1* in thermo-tolerance via regulation of the *Feronia* (*Fer*) homologs (Yin et al., 2018). The CRISPR-*bzr1*-mutant shows impaired production of H₂O₂ in apoplast, reduction in induction of *Respiratory Burst Oxidase Homolog 1* (*RBOH1*), and heat tolerance, while as its overexpression enhances H₂O₂ production and recovery of thermo tolerance.

5.2 | Engineered plants for cold tolerance

Crop plants like tomatoes are chilling sensitive; hence, its fruits are prone to damage by cold stress. The *cbf1* mutants generated by CRISPR/Cas revealed that *C-repeat binding factor 1* (*CBF1*) protects the plant from cold/chilling injury and helps avoid electrolyte leakage (Li et al., 2018). The mutants were found capable of accumulating higher levels of indole acetic acid and hydrogen peroxide; thereby providing cold tolerance in tomato plants (Li et al., 2018). CRISPR/Cas

editing of the japonica rice cultivar for *annexin* (*OsANN3*; a gene encoding Ca²⁺-dependent phospholipid binding protein) was found conferring cold tolerance and enhances its relative electrical conductivity (Shen et al., 2017). CRISPR/Cas9 editing of rice for *stress/ABA-activated protein kinase 2* (*SAPK2*) gene attributes it with resistance to cold stress (Lou et al., 2017). Gene editing by CRISPR/Cas resulted in the development of highly efficient *Ospin5b* mutant (panicle length gene; 53% efficiency), the *gs3* mutant (grain size gene; 66% efficiency), and the *Osmby30* mutant (cold tolerance gene; 63% efficiency) (Zeng et al., 2020). The mutants were found to exhibit enhanced physiological changes corresponding to the above traits respectively.

5.3 | Engineered plants to drought tolerance

Of the different environmental conditions, drought is an important factor that poses a serious threat to the overall survival of the plants (Gollmack et al., 2014; Sánchez-Rodríguez et al., 2010; Xu et al., 2014). It arises as a gradual process under the global climate change conditions and limits plant productivity by affecting different aspects of plant growth ranging from germination to maturity. CRISPR/Cas editing of the plants has improved them for quantitative and qualitative traits towards ensuring global food security. CRISPR/Cas editing of wheat for *dehydration responsive element binding protein 2* (*TaDREB2*) and *ethylene responsive factor 3* (*TaERF3*) improved its tolerance to drought (Kim et al., 2018). The disruption of the *DNA ligase-4* gene prior to gene targeting (GT) by CRISPR/Cas delivered through *Agrobacterium*-mediated transformation increases GT efficiency and led to the production of bi-allelic mutants with high frequency of *ALS* gene (Endo et al., 2016; Mikami et al., 2015). The CRISPR/Cas edited rice mutants having loss function of *SAPK2* (a primary mediator in ABA signaling) were found more susceptible to drought and oxidative stress than the wild counterparts, thus indicate the need for the *SAPK2* gene in imparting drought tolerance (Lou et al., 2017). The CRISPR/Cas editing system was employed to achieve constitutive expression of *auxin-regulated gene involved in organ size* (*ARGOS8*) in maize via replacement of the native *ARGOS* promoter sequence by *GOS2* promoter (Shi et al., 2017). In the developed *ARGOS8-v1* and *ARGOS8-v2* lines, the level of *ARGOS8* transcript was notably higher than in wild plants. Moreover, a remarkable increase in grain yield was shown by the *ARGOS8* variants under drought conditions and without the loss of yield under optimum growth conditions (Shi et al., 2017). In rice, CRISPR/Cas was used to edit the drought-responsive *OsDREB* gene (Hoang et al., 2016) and the salt-sensitive *OsERF922* gene (Liu et al., 2012). The CRISPR/Cas was able to induce inheritable mutations in rice genome for abiotic stress tolerance (Mao et al., 2016). Abiotic stress tolerance in rice cultivars was also achieved by performing editing of the *OsERF109* gene using the CRISPR/Cas system (Mishra et al., 2018). Additionally, rice plants edited for three ERF family members (*OsBIERF1*, *OsBIERF3*, and *OsBIERF4*) showed improved growth under multiple abiotic stresses (Wang et al., 2016). The CRISPR/Cas9 system was used to develop drought-tolerant

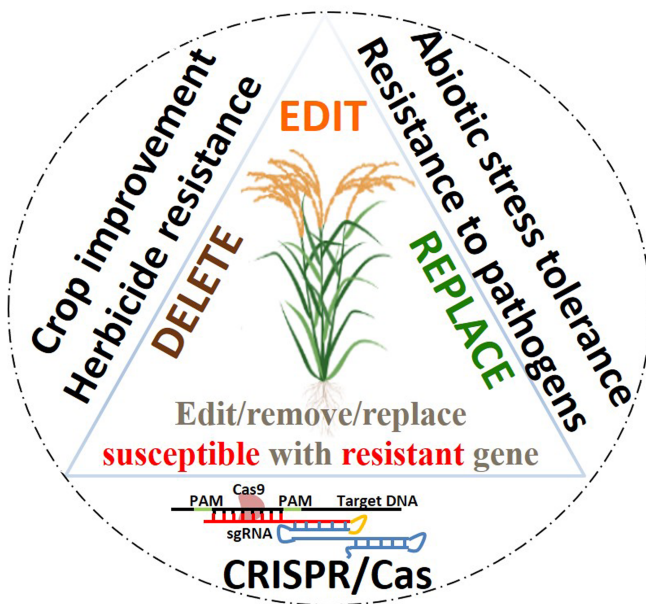


FIGURE 2 Applications of CRISPR/Cas plant genome editing. The figures summarize the fields where CRISPR/Cas editing system has been utilized and has achieved success in terms of overcoming the different abiotic stresses

tomato plants mutant for the *slnpr1* gene (Li et al., 2019). The indica mega rice cultivar MTU1010 *dst* mutant generated by CRISPR-Cas9 editing of *drought and salt tolerance (OsDST)* gene was found having broader leaves with reduced stomatal density towards enhancing leaf water retention under drought stress (Ganie et al., 2021; Santosh Kumar et al., 2020).

Maize crop is mostly cultivated through dry farming techniques; hence, it is important to develop varieties tolerant to drought (Tykot et al., 2006). The *ARGOS8* gene having low expression in maize acts as a negative regulator for ethylene responses. CRISPR-Cas9-mediated editing of ethylene response factor, *ARGOS8* led to a corn variety that shows enhanced tolerance to drought (Shi et al., 2017). The CRISPR-edited lines (CRELs) not only circumvent abiotic stress, but showed improved grain yield as compared to wild type. In maize, *WRKY* genes were found to have abiotic stress-related response. Of the different *WRKY* genes, CRISPR/Cas9 system was utilized to unravel the potential of *ZmWRKY40* gene encoding a transcription factor (C. T. Wang, Ru, et al., 2018). The resultant lines were found imparting maize tolerance to drought.

Reduction in biomass production is the most frequent adverse effect of drought stress on crop plants. In tomato, drought stress exerts severe effect on growth and development that adversely led to reduction in the yield. CRISPR/Cas was employed to decipher the regulatory mechanism of *mitogen-activated protein kinases 3 (SIMAPK3)* gene against drought stress (Wang et al., 2017). The TEY (Thr-Glu-Thy) motifs present in the *SIMAPK3* gene were found playing active role in response to abiotic stress. Knock-out lines in tomato were found imparting resistance to drought. The wild varieties showed fewer symptoms compared to mutant lines having bent stem and severe leaf wilting. The mutant lines also exhibit higher

malondialdehyde, proline, and H_2O_2 levels, which indicates that they suffer from severe membrane damage and oxidative stress (Wang et al., 2017).

5.4 | Engineered plants to salt tolerance

Salt stress constitutes a major factor that constraints production of agricultural crops under coastal or semi-arid regions (Flowers, 2004). The major effects of salt stress include necrosis, premature death of old leaves, and severe disturbance of ions in cells (Julkowska & Testerink, 2015). Plants respond to salt stress through morphological and physiological adaptations, which are the outcome of major shifts in the expression of genes and signaling pathways (Julkowska & Testerink, 2015). The preliminary cellular level changes are quite distinct, which include production of secondary signal molecules like ROS, abrupt changes in Ca^{2+} levels, differential activity of Ca^{2+} /calmodulin-dependent kinase activation, synthesis of ABA, and activation of the salt overly sensitive (SOS) homeostatic signaling pathways (Julkowska & Testerink, 2015; Zeng et al., 2015). Several genes have been verified, by CRISPR/Cas editing, to enhance resistance to salt stress. For example, a multifunctional gene *OsBBS1* in rice was found involved in sensitivity to salt stress and early leaf senescence; in addition, the *OsMIR528* gene was found acting as a positive regulator of salt stress (Ganie et al., 2021; Lan et al., 2019; Mizoi et al., 2012; Sun et al., 2019). The CRISPR/Cas-based targeted mutation verified the regulatory function of the GT-1 element in inducing the expression of the *OsRAV2* gene imparting salt tolerance (Duan et al., 2016). CRISPR-mediated loss of function mutations of *SnRK2* and osmotic stress/ABA-activated protein kinases *SAPK-1* and *-2* genes in rice are reported to confer salinity resistance (Lou et al., 2017). In addition, *OsNAC041* and *OsRR22* genes were also found to increase salt tolerance in rice plants (Ganie et al., 2021, Lan et al., 2019, Mizoi et al., 2012, Sun et al., 2019). In tomato plants, knockout of the *SIMAP3* gene causes significant decrease in the expression of *SILOX*, *SIGST*, and *SIDREB*; thereby inducing salt tolerance to tomato plants (Wang et al., 2017).

5.5 | Engineered plants to herbicide tolerance

Herbicides are chemicals employed to eliminate unwanted plants that grow together with the crop plants. In certain cases, the weeds may be resistant to herbicides and thereby can negatively influence the growth of crop plants. Elimination of weeds/unwanted plants is important, as their presence decreases the crop yield and in turn affects human nutrition. The herbicide tolerance was rendered by the intervention of CRISPR/Cas9 genomic editing tool (Beckie et al., 2019; Li et al., 2015). The point mutation generated by nickase CRISPR/Cas9 (nCas9) variant or nuclease-deficient Cas9 (dCas9) fused to *Petromyza marinus cytidine deaminase (PmCDA1)* resulted in the development mutant lines of rice exhibiting herbicide resistance (Shimatani et al., 2017). Almost prototypical results were also

obtained in soybean to generate chlorsulfuron-resistant mutant lines (Li et al., 2015). The *acetolactate synthase* genes (*ALS1* and *ALS2*) are major targets of genome editing to develop crop varieties resistant to chlorsulfuron (Sun, Zhang, et al., 2016). In maize, *ALS1* and *ALS2* genes were targeted by CRISPR/Cas to produce mutant lines with herbicide resistance (Svitashev et al., 2015). Maize plant resistant to chlorosulfuron can be obtained by using single stranded oligonucleotides as repair templates. Additionally, maize plants having resistance to bispyribac-sodium herbicide are produced by using two gRNAs and a repair template. The NHEJ based intron targeting using chimeric sgRNAs (single-guide RNAs) (Butt et al., 2017), two separate sgRNAs targeting the repair template (Sun, Li, & Xia, 2016) and disruption of DNA ligase 4 implicated in NHEJ repair (Endo et al., 2016), are used to produce rice cultivars resistant to herbicides. In addition, CRISPR/Cas in conjunction with single-stranded oligonucleotides induces herbicide tolerance in flax (Sauer et al., 2016).

6 | CONCLUSIONS AND FUTURE PERSPECTIVES

Amid growing challenges posed by climate change, abiotic stresses impart serious consequences on the growth and development of plants. With serious concerns about reduction in the yield, it often fails in ensuring food security to the global population. Under such circumstances, it becomes imperative to adopt strategic policies regarding attainment of agricultural sustainability to fulfill the need of food for growing population. The above problems have led scientific fraternity to look for alternatives; generating superior cultivars by following traditional breeding approaches or opt for advanced techniques of editing the genome for desired traits that can ensure sustainability in agriculture. Conventional breeding approaches improve progeny for desired traits by attributing it with natural phenotypic and agronomic traits. Identification of traits for combination and selection of elite varieties with such traits improves crop plants for yield, nutritional value, and biotic as well as abiotic stress tolerance. However, conventional breeding approaches face two major bottlenecks: (1) use of marker genes during transformation, and (2) low transformation efficiency of the transgenes. The genome engineering tools have overcome challenges posed by traditional breeding approaches that led to shift from domestication to settled crop production system (Figure 2).

CRISPR/Cas-mediated gene editing in one such approach emerged as major tool for enhancing tolerance of crop plants to different stresses. CRISPR/Cas system has evolved as a tool of high efficiency, specificity, time and cost effectiveness. The CRISPR/Cas system is preferred globally for deciphering gene function for translational utility. The technique has been employed to diversify the crop improvement programs towards mitigation of abiotic and biotic stress challenges. The technique has also been employed to engineer plants for enhancing crop yield, nutritional value and disease resistance and critical traits for food security and sustainable agricultural growth. It has superseded the traditional conventional breeding methods for being effective and highly accurate in performing precise editing of

the genome towards achieving zero hunger goal (one among the 17 sustainable goals) set by United Nations for 2030. Giving credit to its simplicity, specificity, amenability to multiplexing and high efficiency, CRISPR/Cas gene editing system is promising in improving agricultural practices and preventing the spread of invasive species. The genome editing through CRISPR/Cas is widely accepted method in plant biology, paving way for next generation breeding programs. The future strategies include removing the transgene concept, precise and multiple editing protocols that need to be established and tried to produce allied varieties that are resistant to biotic and abiotic stresses and are suffice to fulfill the growing need of sustainability in agriculture in order to ensure global food security.

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AUTHOR CONTRIBUTIONS

Arif T. Jan and Safikur Rahman conceived the idea. Mujtaba A. Bhat, Rakeeb A. Mir, and Vijay Kumar contributed to the writing of the manuscript. Ali A. Shah, Sajad M. Zargar, Safikur Rahman, and Arif T. Jan edited the manuscript and contributed to the compilation of data for table and designing of figures.

CONFLICT OF INTEREST

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

DATA AVAILABILITY STATEMENT

N/a

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