Genome Editing and Abiotic Stress Tolerance in Crop Plants

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Abbreviations

Cas9	CRISPR-associated protein 9
CRISPR	Clustered regularly interspaced short palindromic repeats
DSB	Double-strand breaks
GE	Genome editing/Genetic engineering
GP	Germination percentage
GR	Germination rate
HDR	Homology directed repair
HR	Homologous recombination
MAPK	Mitogen-activated protein kinase
NHEJ	Nonhomologous end-joining
TAL	Transcription activator-like
TALEN	Transcription activation-like effector nucleases
TrugRNA	Truncated RNA
ZFN	Zinc finger nucleases

1 Introduction

Plants are sessile, so they experience various inescapable abiotic stresses in their ecological habitat. In this era of atmospheric change, abiotic stresses such as salinity (Zhang et al. 2017), drought (Moonmoon and Islam 2017), cold (Liu et al. 2019), high temperature (Gabaldón-Leal et al. 2016), and heavy metals (Shahid et al. 2016)

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are being recognized as the most complex environmental disturbances, which reduce the yield and productivity of crop plants and thus are causing food insecurity throughout the world (Bechtold and Field 2018). Plant responses to the adverse effects of abiotic stresses are dependent on the tissue, organ, genotype, ploidy levels, and crop type (Cramer et al. 2011). Abiotic stress commonly promotes overproduction of reactive oxygen species (ROS), which is a signaling molecule that, depending on its concentration, can be toxic to plants, causing damage to cell membranes, protein structure, lipids, carbohydrates, and DNA, damage that ultimately inhibits physiological and metabolic processes in crop plants (Gall et al. 2015). The current most challenging problem is to provide food security to the growing population of ten billion by 2050 (Jaganathan et al. 2018). Global food production needs to be increased by 60–100% above current levels (FAOSTAT 2016). To cope with unfavorable adverse stress conditions, plants activate their physiological and molecular machinery including stress-resistant genes (Hu et al. 2018; Duan and Kai 2012), transcription factors (Guo et al. 2016), secondary metabolites (Selmar and Kleinwachter 2013), antioxidant enzymes (Pandey et al. 2017), and phytohormones (Sah et al. 2016) to survive under abiotic stress.

Conventional breeding techniques have been applied to improve crop production; however, the use of these techniques for enhancing tolerance toward abiotic stresses has been gradually reduced, as it involves complex inheritance as well as high genotype–environment interactions. In addition, these conventional breeding methods were practically unable to overcome the abiotic stresses resulting from climate change (Mushtaq et al. 2018). Therefore, new techniques are needed for further improvement in crop production to meet the current and future food demand under these ever-changing climate conditions (Mishra 2014). Genetic engineering led to a rapid development of crop plants with enhanced stress tolerance, high nutritional value, and yield, but the use of this technique for crop breeding has several limitations over time. The main disadvantage of this technique is the use of foreign DNA in the plant genome without utilizing the plant's own genetic repertoire to achieve the desirable qualitative and quantitative characters. Gradually this technology lost acceptance among consumers, and its use has been reduced, as it creates several risks to the environment and food safety and unsupported health problems because of its non-specificity and unstable nature, which restricted the use of genetically modified crops (Zhang et al. 2018a, b; Stephens and Barakate 2017; Kamthan et al. 2016). Introduction of genetically modified genes or transgenes in a crop plant has been beneficial for global food security, but such genetically modified crops are largely affected by environmental safety concerns. Overexpression of genes through the promoters can cause growth retardation under normal conditions and reduced fruit/seed numbers. This constitutive stress response pathway by overexpressed genes has diverted plant developmental programs, resulting in crop yield loss and fewer benefits for agricultural crops (Marco et al. 2015).

The recent availability of genome editing tools has avoided the limitations of conventional genetically modified or traditional breeding methods and is developing a new age of crop improvement in the field of abiotic stress-tolerant crops (Waltz 2018; Mishra et al. 2018). In comparison to the transgenic approach, which very

often incorporates the phenotype, genome editing methods have become a vigorous technique in the field of crop breeding by the development of defined mutants with the desired traits (Jaganathan et al. 2018). In contrast to genetic engineering, genome editing technology does not involve integration of any foreign DNA into the host plant; as a result, offspring cannot be discriminated from parental plants (Shanmugavadivel et al. 2019). In the genomic field, sequence-specific genome editing is explained as a collection of advanced molecular techniques that would be specific, and efficient for target modification at genomic loci (Gao 2015).

Four kinds of genome editing tools have been used so far: (1) zinc finger nucleases (ZFN), (2) meganucleases, (3) transcription activator-like effector nucleases (TALENs), and (4) clustered regularly interspaced short palindromic repeats (CRISPR) systems (Jain 2015). These techniques modify genomic sequences by using designer sequence-specific nucleases to make double-strand breaks (DSB). The cellular repair system of the plant fixes the double-strand breaks and allows gene insertion and deletion (INDELS) by using nonhomologous end-joining (NHEJ) and homology-directed recombination (HDR) pathways (Jaganathan et al. 2018). Among all genome editing techniques, CRISPR/Cas9 is modern, popular, and the simplest method in plant research (Ma et al. 2016).

The application of genome editing tools (CRISPR/Cas9, dual sgRNA/Cas9, SRSPR sgRNA, and TALENs) has been implemented in different crops for enhancing abiotic stress tolerance such as drought in maize (Shi et al. 2017) and tomato (Wang et al. 2017; Li et al. 2017), salt tolerance in rice (Bo et al. 2019), cold tolerance in rice (Shen et al. 2017a, b), and heavy metal tolerance in rice (Tang et al. 2017). The applications of genome editing tools expand new opportunities in the field of abiotic stress tolerance and aim to improve crop productivity by developing novel varieties. Here, we have summarized the mechanism, potential application, and future implications of genome editing methods for a prospective view for plants. We highlight the advantages of CRISPR-Cas9 over other genome editing tools by describing recent studies on various plants under different abiotic stresses.

2 Types of Crop Plant Genome Editing Tools

To meet the demands of increasing population as well as extreme climatic conditions, ways to improve crop production are required. Therefore, we need a directed, rapid, and low-cost method to improve crop yield and also to develop multi-stress-resistant crop varieties (Xu et al. 2014). Recently, genome editing has come to light as an alternative to improve plant breeding, crop plants, and reasonable food production (Belhaj et al. 2013). Modification of the target region of the genome using genome editing technology has potential advantages over the traditional method of genetic modification, generally done by random insertion events, which most of the time affects the expression level of the transgene (Forsyth et al. 2016). Genome editing can be defined as the alteration of the target genome to illuminate and control gene functions in plant research (Li et al. 2014). In 1993, the first-ever application

of genome editing was implemented in the production of a transgenic tomato commercially in the United States (USA). Subsequently, several preplanned and specific modifications to the genomes of various plants have been accomplished to upgrade genome editing technology and thereby improve crop breeding methods (Zaman et al. 2018).

In genome editing technology, the genome of an organism itself is modified by knocking out or replacing the targeted gene for desired and selected traits, whereas in transgenic approaches biologically nonexistent foreign genes are introduced to the original genome to develop new characters in the existing species (Mushtaq et al. 2018). To date, many genome editing techniques have been implemented in plant molecular biology. These techniques have enabled researchers to make target regions of genes in a DNA sequence-specific manner (Brooks et al. 2014). Methods using zinc finger nucleases (ZFN), transcription activator-like nuclease (TALEN), and clustered regularly interspaced short palindromic repeats (CRISPR)/Cas9 have been applied to modify the targeted plant genome (Jia and Wang 2014) (Fig. 1). These genome editing technologies generally induce double-strand breaks (DSB) or single-strand breaks (SSB), resulting in mutation of the target regions of the genome. The broken ends are then repaired by nonhomologous end-joining (NHEJ) or homologous recombination (HR) methods. Thus by adopting a gene knockout, knockin, or replacement strategy, site-directed mutagenesis-based genome editing is induced at the target regions of the genome, which results in modification of several morphological, physiological, and enhancement tolerance/resistance

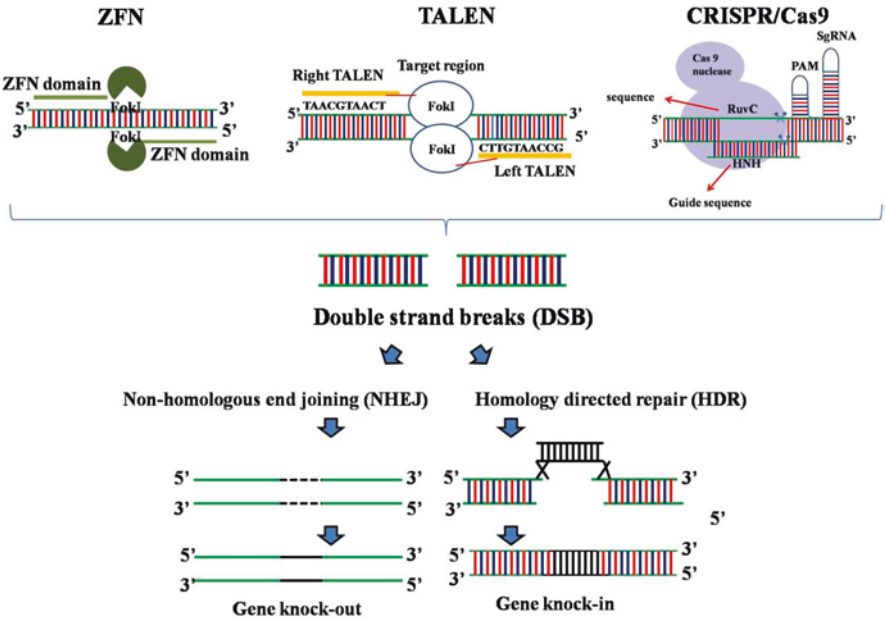

Fig. 1 Schematic representation of generalized genome editing mechanism in crop plants

characters along with the growth and development of different crop plants (Zhang et al. 2017). Modification of the genetic information of plants in an accurate and specific way will not only help us to study the gene function and biological mechanisms but also will help to create various novel phenotypes including enhanced yield and stress-tolerant crops. In this regard, genome editing technology has emerged as an advanced tool for improvement of crop production under abiotic stress (Petolino 2015).

2.1 Zinc Finger Nuclease

Zinc finger nuclease (ZFN) is generally used to cut a target DNA site that is later applied to error-prone nonhomologous end-joining (NHEJ), resulting in mutagenesis of the specific site. ZFNs have been used to modify endogenous genes in a wide spread of organisms and cell types (Urnov et al. 2010; Joung and Sander 2013). Various kinds of genomic alterations such as mutations, deletions, insertions, inversions, duplications, and translocations can be introduced with ZFNs (Fig. 2), which provides researchers with exceptional tools to perform genetic manipulations (Joung and Sander 2013). Fusion of ZFNs consisting of zinc finger protein domains, capable of sequence-specific DNA binding, and a nuclease domain is generally used for identification of protein domains, each recognizing approximately 3 bp DNA (Petolino 2015).

In its first application, ZFN enzymes in plants used a reporter sequence newly incorporated into the plant genome to separate ZFN-derived mutants (Tzfira et al. 2012). Afterward, several site-specific mutations using ZFN constructs were stably integrated into the plant genome (Osakabe et al. 2010; Zhang et al. 2010). Although

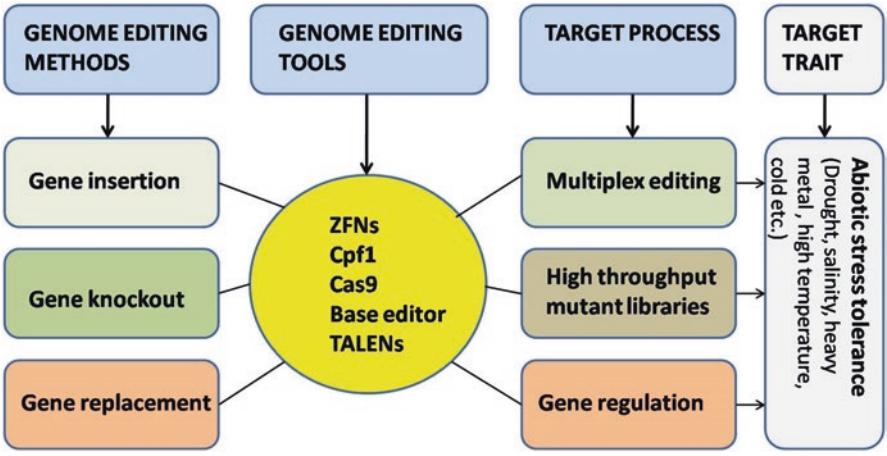

Fig. 2 Schematic depiction of genome editing methods, tools, target process, and target trait in developing abiotic stress-tolerant crop plants

in some cases short deletions count for 80% of the ZFN-induced mutations, in others nucleotide substitutions were 70% of all mutations induced (Lloyd et al. 2005). The design and application of ZFNs include modular design, assembly, and development of zinc fingers against specific DNA sequences, followed by linking of single ZFs in the direction of targeting larger sequences. In recent years, zinc finger domains have been created to identify a large number of triplet nucleotides, which allowed the selection and joining of zinc fingers in a sequence that would permit recognition of the target sequence of interest (Kamburova et al. 2017).

The first ZFN-mediated gene knockouts were implemented in the tobacco acetolactate synthase gene (ALS) known as SuRA for the development of herbicide-resistance plants (Maeder et al. 2008; Podevin et al. 2012). In *Arabidopsis* the ZFN technology has been used to efficiently cleave and stimulate mutations at an endogenous target gene, ABA-INSENSITIVE4 (ABI4). This gene encodes a member of the ERF/AP2 transcription factor family and has a role in regulating abscisic acid (ABA), which controls a number of agronomically important traits, including plant responses to abiotic stress and seed development. This ZFN-based genome modification results in mutation of the target gene ABI4 at a rate of approximately 0.26–2.86% in *Arabidopsis* somatic cells and transmission of the induced mutation in the target gene to subsequent generations (Osakabe et al. 2010).

So far gene modification by ZFN has been successfully implemented in soyabean (Curtin et al. 2013), *Arabidopsis thaliana* (Zhang and Voytas 2011; Qi et al. 2013; Li et al. 2014), maize (Shukla et al. 2009), and tobacco (Townsend et al. 2009; Jia and Wang 2014). However, the ZFN-based technology has a number of limitations from the complexity and high cost of protein domain construction for each particular genome locus and the chances of defective cleavage of target DNA from single nucleotide substitutions or unsuitable interaction between domains. Therefore, the search for new methods for genome editing continued and led to the development of new tools for genome editing: TALENs (transcription activator-like effector nucleases) and CRISPR/Cas9 (clustered regulatory interspaced short palindromic repeats) (Nemudryi et al. 2014).

2.2 Transcription Activator-Like Effector Nucleases (TALEN)

The idea of TALENs development comes from the study of bacteria of the genus *Xanthomonas*. These bacteria are pathogens of crop plants such as rice, pepper, and tomato, causing remarkable economic damage in agriculture, which led to their in-depth study. These bacteria generally secrete effector proteins (transcription activator-like effectors, TALEs) to the cytoplasm of plant cells and affect processes in the plant cell that increases the liability of the cells to the pathogen (Nemudryi et al. 2014). TALENs are generally used to introduce mutation by means of homologous recombination (HR) and nonhomologous end-joining (NHEJ). The homologous donor DNA is used as a template to restore the double-strand break (DSB),

resulting in gene insertion or replacement. Then, the broken ends are joined by NHEJ. Frequently small deletion or insertions are introduced at the junction of the newly rejoined chromosomes (Du et al. 2016) (Fig. 2).

Transcription activator-like (TAL) effectors of *Xanthomonas oryzae* help to reduce the severity of the bacteria *Xanthomonas* by transcriptionally activating the specific rice disease susceptibility (S) gene (Yang et al. 2006; Antony et al. 2010). Therefore, TALEN technology has been applied to edit the specific S gene in rice to counter the virulence strategy of the bacteria *Xanthomonas*. This engineered genome modification results in resistance to bacterial blight, a destructive disease in crops. A combination of TAL effector nucleases (TALENs)—fusion proteins derived from the DNA recognition repeats of unaffected TAL effectors and the DNA cleavage domains of Fok I (Christian et al. 2010; Li et al. 2010; Miller et al. 2010)—have been used to create site-specific gene modifications in plant cells (Mahfouz et al. 2011; Cermak et al. 2011; Li et al. 2012).

TALENs have also been used to modify the genome of the model plant, *Arabidopsis thaliana*. Here TALENs are used to target five *Arabidopsis* genes, namely, ADH1, TT4, MAPKKK1, DSK2B, and NATA2. In pooled seedlings expressing the TALENs, the somatic mutagenesis frequencies ranges from 2% to 15%. However, after modification of the genes by using TALENs, the somatic mutagenesis frequencies rise to 41–73% in individual transgenic plant lines expressing the TALENs. Additionally, a TALEN pair targeting a randomly duplicated gene induced a 4.4-kb deletion in somatic cells (Christian et al. 2013). In potato tubers, cold temperature usually stimulates the accumulation of reducing sugars. At the onset of high temperature, these reducing sugars react with free amino acids to give brown, bitter-tasting products with high levels of acrylamide, a potential carcinogen. To control the accumulation of reducing sugars, RNA interference (RNAi) technology was used to silence the vacuolar invertase gene (VInv). This gene encodes a protein that breaks down sucrose to glucose and fructose. Because RNAi often results in incomplete gene silencing, the transcription activator-like effector nucleases (TALENs) were applied to knock out the VInv gene within the commercial potato variety, Ranger Russet. For this, transiently expressing transcription activator-like effector nucleases (TALENs) are designed to bind and cleave specific DNA sequences in the Vinv locus. TALENs successfully result in complete VInv knockout lines without integrating any foreign DNA. The new potato lines have significantly lower levels of reducing sugars and acrylamide in heat-processed products (Clasen et al. 2015).

In comparison to ZFN, researchers have shown much interest in TALEN as they can be very easily and rapidly designed using a simple protein–DNA code. This protein–DNA code relates modular DNA-binding TALE repeat domains to individual bases at a specific binding site (Joung and Sander 2013). As ZFN and TALEN require considerable time and effort because of the difficulties in protein design, synthesis, and validation, the CRISPR/Cas9 system is widely used for genome editing as it has simplicity, design flexibility, and high efficiency (Wang et al. 2017).

2.3 Clustered Regularly Interspaced Short Palindromic Repeat (CRISPR/Cas9)

The CRISPR locus was first observed in *Escherichia coli* (Ishino et al. 1987). Currently, it is known to be present in about 84% of Archaea and 45% of Bacteria (Grissa et al. 2007). The CRISPR system is an arrangement of short repeated sequences separated by spacers with unique sequences. The CRISPR is generally found on both chromosomal and plasmid DNA. The spacers are commonly derivatives of the nucleic acids of viruses and plasmids (Rath et al. 2015). The CRISPR/Cas9 system consists of a Cas9 endonuclease that is a derivative of *Streptococcus pyogenes*. In this process a chimeric single guided RNA is used to direct Cas9 to a specific DNA sequence in the genome, which results in a DNA double-strand break in the specific locus through Cas9. The DSB is repaired through either endogenous nonhomologous end-joining or through the high-fidelity homology-directed repair (HDR) pathways. NHEJ generally induces small insertions or deletions at the repair junction whereas HDR stimulates programmed sequence correction as well as DNA fragment insertion (Shi et al. 2017).

CRISPR activity is generally regulated by a set of CRISPR associate (Cas) genes, usually found close to the CRISPR. The Cas genes code for proteins essential to the immune response. The CRISPR-Cas mediated defense process functions in three stages. The first stage is called adaptation, which leads to insertion of new spacers in the CRISPR locus. The next step is known as expression, where the system is prepared for action by expressing the *cas* genes and transcribing the CRISPR into a long precursor CRISPR RNA (pre-crRNA). Subsequently, the pre-crRNA is organized into mature crRNA by Cas proteins and accessory factors. In the third stage, the target nucleic acid is identified and eliminated by the co-actions of crRNA and Cas proteins: this is the last stage of CRISPR-mediated action, known as interference (Rath et al. 2015).

The gRNA/Cas9 technology has used for targeting the *Arabidopsis thaliana PDS3* (*PHYTOENE DESATURASE*) gene in *Arabidopsis* mesophyll protoplasts, which are freshly isolated leaf cells without cell walls. The protoplast transient expression system supports highly efficient DNA co-transfection and protein expression. The application of gRNA/pcoCas9-mediated genome editing has also been extended to other plant systems, such as tobacco (*Nicotiana benthamiana*) protoplasts. Significantly higher mutagenesis frequencies were observed, that is, 37.7% and 38.5%, for targets 1 and 2, respectively, by targeting *NbPDS* (ortholog of AtPDS3) at two different sites than in *Arabidopsis*. Interestingly, the gRNA/pcoCas9-induced mutagenesis often led to significant DNA deletions or insertions but only rare single nucleotide (nt) substitutions in tobacco cells as in animal and human cells showing relatively high mutation rates (Li et al. 2013).

This system has also been successfully exploited in rice protoplast cells transformed with Cas9/sgRNA constructs targeting the promoter region of the bacterial blight susceptibility genes, OsSWEET14 and OsSWEET11 (Jiang et al. 2013; Miao

et al. 2013). The CRISPR/Cas9 SSN (C-ERF922) was used to target the OsERF922 gene, ERF, and TF genes in rice to enhance blast disease resistance (Wang et al. 2016a, b). By using the CRISPR/Cas9 system the role of OsMIR528 in rice was identified as a positive regulator in salt stress, targeting miRNAs (Zhou et al. 2017; Shanmugavadivel et al. 2019). Xu et al. (2016) have used the CRISPR/Cas9-mediated multiplex genome editing approach in rice. Here three genes, namely, GW2, GW5, and TGW6, were selected, and mutation in any one of these three genes resulted in significant increase of grain weight, which is considered as one of the most crucial quantitative traits in rice production (Xu et al. 2016).

To enhance the specificity and reduce the off-target effects of CRISPR-Cas system, approaches such as using Cas9 nickase, Cas9n, and dCas9 (mutated version of Cas9), careful design, and gRNAs truncated at the 5′-ends (trugRNAs) have been adopted to create the site-directed modifications in plants (Osakabe et al. 2016; Mushtaq et al. 2018).

So far, genome editing using CRISPR/Cas9 has been successful for various crop plants such as tomato (Wang et al. 2017; Li et al. 2019a, b), rice (Lou et al. 2017), and maize (Shi et al. 2017) for enhancement of drought tolerance. This technology has also been implemented to increase cold tolerance in rice (Shen et al. 2017a, b) and *Arabidopsis* (Li et al. 2016), and it has been applied under salinity and heavy metal stress in rice (Bo et al. 2019; Tang et al. 2017). Studies on the recent application of CRISPR/Cas9 in various crop plants for the enhancement of abiotic stress tolerance with improved morphological, physiological, and yield characteristics are depicted in Table 1.

3 Uses of CRISPR/Cas9 in Enhancing Abiotic Stress Tolerance in Crops

Clustered regularly interspaced short palindromic repeats associated endonuclease Cas9 (CRISPR/Cas9) is an immune system obtained from the microbes (Hryhorowicz et al. 2017). The CRISPR/SgRNA:Cas9 has been successfully implemented in diverse plant families such as Fabaceae (Cai et al. 2018; Jacobs et al. 2015; Du et al. 2016), Poaceae (Kim et al. 2018; Howells et al. 2018; Minkenberg et al. 2016), Malvaceae (Long et al. 2018; Gao et al. 2017), Asteraceae (Ynet and Yilancioglu 2018), and Solanaceae (Anderssona et al. 2018; Brooks et al. 2014). Many research articles discuss the application of the CRISPR-Cas9 system by knocking out a particular reported gene that is which involved in the abiotic stress-tolerant mechanism in plants. The customized CRISPR/Cas9 has become the easiest way of transformation in plants within less time (Belhaj et al. 2015; Bortesi and Fischer 2015). Applications of CRISPR in various crop species under different abiotic stresses are described briefly next.

Table 1 List of target genes manipulated via genome editing in different crop plants for enhancing abiotic stress tolerance

Genome editing methods	Crop plants	Target gene	Stress	Key findings	References
CRISPR/Cas9	Maize (*Zea mays*)	*ARGOS8*	Drought	Drought-tolerant breeding, reduced ethylene response, increase growth and development, and increased grain yield by five bushels per acre under flowering stress conditions	Shi et al. (2017)
	Tomato (*Solanum lycopersicum*)	*SlMAPK3*	Drought	Protects cell membranes from oxidative damage and regulates transcription of stress-related genes, thereby increasing growth and development	Wang et al. (2017)
	Tomato (*Solanum lycopersicum)* Cv. Ailsa Craig	*SlNPR1* mutant	Drought	In comparison to wild type, the *SlNPR1* mutant plants showed reduced drought tolerance, negative regulation of stomatal closure, increased electrolytic leakage, H_2O_2, and MDA content, decreased expression of antioxidant enzymes such as APX, SOD, POD, lower expression level of drought-related genes	Li et al. (2019a, b)
	Rice (*Oryza sativa* L.) Cv. Japonica	*OsSAPK2*	Drought	Enhanced drought tolerance, detoxification of ROS by increasing activities of antioxidant enzymes	Lou et al. (2017)
	Rice (*Oryza sativa* L.)	*OsNAC041*	Salinity	Salt-resistant plants with increased seed germination, growth, decreased level of ROS and MDA accumulation	Bo et al. (2019)
	Rice (*Oryza sativa* L.)	*OsAnn3*	Cold/4–6 °C for 3 days	Tolerance to cold stress, increased survivability up to 81.1% decreased electrical conductivity	Shen et al. (2017a, b)

	Rice (*Oryza sativa* L.) Cv. Huazhan, Cv. Longke 638S	*Osnramp5*	Heavy metals	Lower accumulation of manganese (Mn) and cadmium (Cd) in rice plants, no effect of growth, yield, and agronomic traits	Tang et al. (2017)
	Rice (*Oryza sativa* L.)	*OsPDS, OsMPK2, OsBADH2*	Multiple abiotic stress	Three rice genes showed tolerance capacity against various abiotic stress factors	Shan et al. (2013)
	Rice (*Oryza sativa* L.)	*OsMPK5*	Multiple abiotic stress	Plant exhibited various abiotic stress tolerance and disease resistance	Xie and Yang (2013)
	Rice (*Oryza sativa* L.)	*OsMPK2, OsDEP1*	Multiple abiotic stress	Yield under different abiotic stress conditions	Shan et al. (2014)
	Rice (*Oryza sativa* L.)	*OsDERF1, OsPMS3, OsEPSPS, OsMSH1, OsMYB5*	Drought	Plant showed drought resistance	Zhang et al. (2014)
	Rice (*Oryza sativa* L.)	*OsHAK-1*	Heavy metal stress	Plant showed low cesium accumulation	Cordones et al. (2017)
	Rice (*Oryza sativa* L.)	*OsPRX2*	Heavy metal stress	Potassium deficiency tolerance	Mao et al. (2018)
	Arabidopsis thaliana	*OST2* (OPEN STOMATA 2) (AHA 1)	Osmotic stress	Increased in the stomatal closure in response to abscisic acid (ABA)	Osakabe et al. (2016)
CRISPR sgRNA	Rice (*Oryza sativa* L.)	*OsAOX1a, OsAOX1b, OsAOX1c,OsBEL*	Multiple abiotic stress	Plant exhibited various abiotic tolerance mechanisms	Xu et al. (2015)
Dual-sgRNA/ Cas 9	*Arabidopsis thaliana*	*MIR169a*	Drought	Increased in the yield under drought stress condition	Zhao et al. (2016)
TALEN	Barrelclover (*Medicago truncatula*) v. R108	*MtCAS31*	Drought	Reduced negative effects of drought stress on symbiotic nitrogen fixation. MtCAS31 protects MtLb120-1 from the damage of drought stress	Li et al. (2018)

3.1 Drought

Compared to other abiotic stress factors, water deficit or drought is the most devastating stress affecting plant growth and yield (Zhang et al. 2018a, b). Drought stress normally occurs when the transpiration rate is higher than the uptake of water by the roots (Salehi-lisar et al. 2012). According to a global scale, drought stress decreases cereal production by 9–10% (Lesk et al. 2016). Drought or water deficit hinders plant growth and development by decreasing water uptake of the plant cells with decrease in the cell volume and cell wall size and unfavourably affects many physiological and biochemical responses (Li et al. 2019a, b). Drought stress also interferes with the photosynthetic process by reducing intercellular CO_2 concentration (Ci), chlorophyll *a/b* degradation, hydrolysis of chloroplast protein, and reducing of leaf pigments (Liang et al. 2019). Under drought stress plants indemnify by acquiring immobile nutrients, which alter the accumulation of beneficial metabolites such as proline and soluble sugar (Muler et al. 2014). Exposure to drought stress leads to accumulation of ROS, oxidation of amino acids, DNA nicking, lipid peroxidation, etc. (Nezhadahmadi et al. 2013). The causes of drought stress are various relevant conditions such as inadequate rainfall, low moisture quality of soil, evaporation demand, and imprudent water utilization (Salehi-Lisar and Bakhshayeshan-Agdam 2016). Plants that develop tolerance capacity normally limit the number and area of leaves, which results in yield loss under drought stress (Akhtar and Nazir 2013). This stress negatively affects crop yield (Fahad et al. 2017). Daryanto et al. (2016) reported that under drought stress both maize and wheat experienced crop yield reduction as much as 20% and 39%, and also showed fertilization failure during the reproductive stage (Daryanto et al. 2016). Farooq et al. (2017) presented that under drought stress, an important food crop, cowpea, can reduce yield by 34–68% (Farooq et al. 2017). Ten days of drought stress on 35-day-old rice seedlings and 10 days of drought stress at the reproductive stage showed reduction of grain in four cultivars: Swarna Sub1 (46.07%), Nagina 22 (19.71%), NDR 97 (20.32%), and NDR 102 (24.94%) (Singh et al. 2018). Wei et al. (2018) have described that soybean plants on which was imposed drought stress at the vegetative growth stage showed reduction as great as 70–82% (Wei et al. 2018). Thus, application of the transgenic-based approach by introducing TFs to produce genetically engineered plants has reached negative perception because of the limitation of greenhouse trials and the additional cost.

To overcome this issue, nowadays genome editing is the acceptable alternative used in plant breeding (Lamaoui et al. 2018). In the past few years, efficient genome editing technologies have come to light in the research field by the rapid manipulation of DNA sequences and developing drought-tolerant germplasm by editing natural chromosomal context (Shi et al. 2017). Ethylene is responsible for the plant abiotic stress condition, which confers water deficit and high temperature (Müller and Munné-Bosch 2015). *ARGOS8* is a negative regulator of ethylene response that modulates ethylene transduction under drought stress when it is overexpressed in maize plants (Shi et al. 2015). Shi et al. (2017) used CRISPR-Cas9-enabled advanced breeding technology to generate maize lines carrying *ARGOS8* genome-edited variants, which increased maize grain yield and tolerance phenotypes under

drought stress conditions. An RNA-guided Cas9 endonuclease was used to generate DNA double-strand breaks in a site-specific manner for integrating the GOS2 PRO in to the upstream region of *ARGOS8* via homology-directed DNA repair to moderate constitutive expression of *ARGOS8*. The genome-edited plants showed higher expression of *ARGOS8* relative to wild-type (WT) controls. Under field conditions both the ARGOS8-V1/V2 variants showed grain yield of approximately five bushels per acre compared to the wild type. Also, early cessation of grain filling, less grain moisture, plant height, and ear height increased up to 2.6 cm and 3.2 cm in the two variants, respectively (Shi et al. 2017). Wang et al. (2017) aimed to study the function of *SlMAPK3* in tomato plants by using CRISPR/Cas9-mediated *SlMAPK3* mutants to find possible regulating mechanisms for drought tolerance. Both *SlMAPK3* mutants and WT plants were kept under drought stress of 23–25 °C with photoperiod 16:8 h light/dark, withheld from 6-week-old tomato plants for 5 consecutive days, and treated with 25% (w/v) PEG 6000 to analyze drought tolerance and to explore the regulatory mechanism. WT plants showed fewer wilted leaves compared to *SlMAPK3* mutant plants. Ion leakage was 70–83% higher than WT plants, with higher MDA content, more proline content, and H_2O_2 content significantly higher relative to WT control plants. All these elevated contents lead to damage to membranes by accumulating ROS and disturbing membrane integrity and stability. Also, activities of antioxidant enzymes in all mutant lines were significantly lower than in WT. Taken together, all these data reveal that *SlMAPK3* is a positive regulator of drought stress. A control line with expression of *SlMAPK3* is involved in drought response in tomato plants by protecting cell membranes from oxidative damage and modulating transcription of stress-related genes (Wang et al. 2017). Li et al. (2019a, b) used CRISPR-associated protein-9 nuclease (Cas9) technology to predict the function of *SlNPR1* and generated *SlNPR1* mutant tomato plants to compare with the WT tomato plants to analyze physiological and molecular mechanisms under drought stress. *SlNPR1* mutant plants showed seriously wilted leaves, bent stems, lower survival rate, and more stomatal closure compared to WT plants. Electrolyte leakage was 55–63%, and H_2O_2 accumulation was 230–221 $nmol^{-1}$ g^{-1} FW, with higher MDA level compared with WT. Under drought conditions, loss of SlNPR1 function in SlNPR1 mutants led to downregulation of antioxidant enzymes or the antioxidant genes *SlGST*, *SlDHN*, and *SlDREB*. These results suggest that *SlNPR1* might be involved in abiotic stress responses, such as drought stress (Li et al. 2019a, b). The overall studies confirm that by applying CRISPR/Cas9 technologies, there is remarkable potential to improve drought tolerance in important crop plants such as maize and tomato. Thus, this CRISPR/Cas9 technology can be implemented in other crops such as rice and wheat.

3.2 Salinity

Among all stresses, salinity is a vital stress reducing viable agricultural land and the demand for food crops (Gupta and Huang 2014). According to the FAO, salinity stress affects 6% of agricultural land worldwide, which exhibits serious limiting

factors for plant growth and productivity (Parihar et al. 2015). Salinity stress is of two types: (1) hyperosmotic stress and (2) hyperionic stress, which have different effects on plants under salt stress. In hyperosmotic stress, plants lose water from the root system and leaves, which changes various physiological and morphological characters including destroying the ability to detoxify abiotic stress, decrease antioxidant mechanism, impair photosynthetic activity, and decrease stomatal aperture. Under the hyperionic condition, plants uptake high salt that inhibits the intake of essential minerals such as phosphorus (P), potassium (K^+), nitrogen (N), and calcium (Ca^{2+}) (Gupta et al. 2015). This mineral deficiency induces disturbances in osmotic balance and enzymatic activity (Ashraf et al. 2018). Salinity notably affects fruiting, flowering, seed growth, and seed germination (Rai et al. 2013). The yield in crop plants including several growth parameters such as plant height, fresh weight yield, and biomass production are severely affected by salinity stress (Semiz et al. 2012).

The NAC transcription factor family has a key role in altering the number of plant metabolic pathways under abiotic stress such as drought and salinity (Xu et al. 2013; Lee et al. 2017; Shen et al. 2017a, b). Bo et al. (2019) created an OsNAC041 mutant by using the CRISPR/Cas9 method to determine the specific function of rice NAC transcription factor coding gene OsNAC041 under salt treatment. Under 150 mmol/l NaCl treatment, shoots of the wild-type seedling were taller than mutant plants. The wild-type seedling remained alive, whereas almost all the mutant seedlings died. The O_2^- and H_2O_2 levels also revealed a significant increase in ROS accumulation and MDA content in the mutants compared with the wild type.

OsNAC041 mutants affected the membrane protection system by decreasing activities of sediment oxygen demand (SOD), photochemical oxygen demand (POD), and chloramphenicol acetyl transferase (CAT), thereby weakening salt tolerance. These findings provided evidence that OsNAC041 has an important role in salt resistance in rice (Bo et al. 2019). So far, much less work has been carried out on crop plants using genome editing tools to improve salt tolerance. Plant breeders can use CRISPR/Cas9 to improve salt stress tolerance and to understand the physiological responses of plant growth and development.

3.3 Heavy Metals

Heavy metals are nonbiodegradable, with atomic mass greater than 20 and density greater than 5 g/cm^3, and have cytotoxic, genotoxic, and mutagenic effects on living organisms such as plants (Rascio and Navari-Izzo 2011). Toxic heavy metals evoke stress by accumulating ROS, promote DNA damage, or disturb the DNA repair mechanism, and also hinder membrane functional integrity, protein function, and activities (Tamás et al. 2014). Heavy metal pollution can cause crop growth stress, affecting crop production and the quality of crops as well as affecting human health after entering the human body through the food chain (Lei et al. 2015).

Cadmium (Cd) is a heavy metal that is highly toxic for most living organisms (Clemens et al. 2013). A recent survey showed that Cd concentration is high in rice grains. So, controlling Cd accumulation in rice grains is important for food safety and the health of people who consume rice as a daily food in their diet (Jallad 2015). CRISPR/Cas9 has been successfully used to minimize the Cd content in rice grains (Tang et al. 2017). Tang et al. (2017) have developed a low-Cd new Indica rice line by knocking out the gene OsNRamp5 using the CRISPR/Cas9 system. Under toxic conditions of 2.5 μM Cd, the *Onramp5* mutant rice lines showed lower Cd concentration, less than 0.05 mg/kg, compared with the grain of WT plants at 0.33–2.90 mg/kg. Also, low Cd accumulation led to decreased rescue of reduced growth in mutant rice lines relative to WT plants (Tang et al. 2017). However, studies on different crop plants under heavy metal stress and application of CRISPR/Cas9 technology to improve crop tolerances to heavy metals are relatively few.

3.4 Cold Stress

The yield of crops, and their quality and distribution, have been affected by cold stress in various parts of the world. Cold stress generally affects leaf photosynthesis and biomass accumulation, which are the main sources of grain yield (Liu et al. 2019). Yield loss caused by low temperatures is a major restriction on rice cultivation not only in areas at high latitudes or high altitudes but also in tropical countries such as the Philippines and Thailand. Rice plants have a lower threshold temperature (10–13 °C) for cold damage during the early stages of development (germination and vegetative) (Cruz et al. 2013). To enhance cold tolerance in various crops, several transgenic techniques have been applied routinely. The main aim is to identify the novel gene that has the ability to increase cold tolerance (Shen et al. 2017a, b).

Shen et al. (2017a, b) suggested that the rice annexin gene *OsAnn3* was involved in cold tolerance by the knocked-down *OsAnn3* gene in Japonica rice variety Taipai.309 via CRISPR/Cas9 mediated genome editing. Under 4–6 °C for 3 days cold treatment and then return to normal growth conditions, after 10 days wild-type plants showed a survival rate up to 75–81.1%, whereas in T1 mutant plants the survival rate was 55.5%. Electrical conductivity levels increased in the T1 mutants compared to wild-type plants. These results indicated that the knockdown of *OsAnn3* in rice significantly decreases cold tolerance, and also it shows that the presence of *OsAnn3* can enhance plant tolerance under cold stress (Shen et al. 2017a, b). Li et al. (2016) have demonstrated that two *Arabidopsis* glycotransferase genes, *UG79B2* and *UGT79B3*, are involved in cold stress under the regulation of CBF1 by using CRISPR/Cas9 and RNAi technology. Twelve-day-old *Arabidopsis* wild-type, overexpressed *UGT79B2/B3OE* plants and RNAi, Cas9 mutant lines *ugt79b2/b3* were exposed under 4 °C cold conditions. At lower temperature, −12 °C, both *ugt79b2/b3* mutant lines and the wild type turned completely white, with more ion loss, less survivability, whereas overexpressed *UGT79B2/B3OE* plants showed 25% survival rate, less ion leakage, higher antioxidant capacities, and accumulated

anthocyanin for scavenging ROS, which led to enhanced tolerance under cold stress (Li et al. 2016). The use of genome editing tools, especially the CRISPR/Cas9 system, has been able to identify various cold stress-related genes in plants such as rice and *Arabidopsis* through overexpression and mutation.

4 Conclusion

Genome editing is revolutionizing crop breeding to the next generation for its several useful features such as ease of use, accuracy, simplicity, high specificity, and tolerable target effects. Genome editing as an advanced molecular biology technique can produce precisely targeted modifications in any crop plant. Given the availability of a variety of genome-editing tools with different applications, it is important to consider the optimal system for a given species and purpose. With the progress already made in the development of genome-editing tools and the development of new breakthroughs, genome editing promises to have a key role in accelerating crop breeding and in meeting the ever-increasing global demand for food. Moreover, the exigencies of climate change call for great flexibility and innovation in crop resilience and production systems. Application of genome editing tools in improving crop plant tolerance to abiotic stress, yield enhancement, grain quality, nutritional value, and other important agronomic traits will be prominent areas of work in the future. To date, most work in using genome editing technology has been preliminary and needs further improvement to efficiently utilizing the platform that leads to increasing on-target efficacy, and thereby the global food security of the ever-growing population of the world.

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