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Review Article

CRISPR/Cas-based genome editing to improve abiotic stress tolerance in plants

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ABSTRACT:
Climate change is affecting agriculture in a number of ways, such as changing water distribution, daily temperatures and salinity patterns. In this regard, plant breeding innovations and genetic engineering approaches to improve abiotic stress tolerance are necessary to avoid a decline in crop yields caused by climate change during the 21st century. In the last few years, genome editing using the CRISPR/Cas system has attracted attention as a powerful tool that can generate hereditary mutations. So far, only a few studies using the CRISPR/Cas system have been reported to improve abiotic stress tolerance, but they have clearly suggested its effective role for future applications in molecular breeding to improve abiotic stress tolerance. Accordingly, the CRISPR/Cas system application is introduced in this mini-review as a way to improve abiotic stress tolerance. Although editing efficiency and target discovery for plant CRISPR/Cas systems require further improvement, CRISPR/Cas systems will be the key approach to maintaining global food security during climate change.

Keywords:
CRISPR/Cas system, climate change, drought, salt, high temperature

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INTRODUCTION

Global warming and global climate change have affected water distribution, daily temperatures, salinity patterns and nutrient availability in soil, results which have greatly affected crop growth and caused more than 50% of worldwide losses in the yields of major crops every year (Gusta 2012; Verma & Deepti 2016). Since global food demand is expected to approximately double by the 2050s (Tilman *et al*. 2011), increasing crop production to meet rising demands against the threats of global warming and global climate change remains a challenge in agriculture. Therefore, there is need to understand the climatic factors influencing agricultural production and develop climate-resilient crops using modern breeding technologies.

Although conventional breeding remains an essential technique to improve crops, it does not lead to significant improvement in climate resilience due to complex inheritance and high genotype × environment interaction (Bhat *et al*. 2016). In addition, an approach combining genetic engineering and omics technologies has been introduced as a suitable strategy because genomics makes it possible to directly study the genotype and its relationship with the phenotype (Tester & Langridge 2010). However, commercialisation of genetically modified (GM) crops and advancements in GM crops are limited because of public concerns about their safety and efficacy (Prado *et al*. 2014; Raman 2017). Recently, the clustered regularly interspaced short palindromic repeats (CRISPR)/CRISPR-associated protein 9 (Cas9) system has emerged as a powerful genome editing tool to efficiently induce targeted mutations in a genome by removing, adding or altering sections of the DNA sequence (Bortesi & Fischer 2015). Since the CRISPR/Cas9 system has been established for use in *Arabidopsis thaliana*, rice (*Oryza sativa*), *Nicotiana benthamiana* and wheat (*Triticum aestivum*) (Li *et al*. 2013; Nekrasov

et al. 2013; Shan *et al*. 2013), continuous improvements in the system have allowed us to generate transgene-free edited plants (Hussain *et al*. 2018). In addition, the CRISPR/Cas system has been effectively employed to understand the molecular basis of abiotic stress tolerance in crops. Increasing knowledge regarding the molecular mechanisms underlying abiotic stress responses in crops and advancements in the CRISPR/Cas system have provided new opportunities to generate climate-resilient crops. The present article represents a focused mini-review of papers on applying the CRISPR/Cas system to improve abiotic stress tolerance.

CRISPR/CAS-BASED GENOME EDITING FOR IMPROVING ABIOTIC STRESS TOLERANCE IN PLANTS

Abiotic stresses, including drought, extreme temperatures (high and low) and soil salinity are important consequences of climate change and the leading cause of yield loss and decreasing crop productivity. Therefore, abiotic stress-tolerant crops are needed to ensure global food security under conditions of anthropogenic climate change. Although only a few studies have introduced the CRISPR/Cas system to develop crops with increased abiotic stress tolerance (Table 1), these studies clearly show the potential and effective role of the CRISPR/Cas system for future applications in molecular breeding to enhance abiotic stress tolerance.

IMPROVED DROUGHT STRESS TOLERANCE IN HIGHER PLANTS USING THE CRISPR/CAS SYSTEM

Climate change is expected to increase the intensity and frequency of drought conditions due to disturbance in rainfall patterns (Cai *et al*. 2014). Among the various adverse effects of drought, its influence on agriculture is the most significant and direct. In China, annual direct economic losses to agriculture from drought were approximately 32 billion yuan in the period from 1949 to 2013 (Chou *et al*. 2019). Under drought stress, plants display various morphological (e.g., reduced leaf area), physiological (e.g., water-use efficiency and stomatal movement), and biochemical (e.g., accumulation of osmolytes and enzymatic and non-enzymatic antioxidants) characteristics (Fang & Xiong 2015). Among these responses, the earliest reaction to drought in most plants is stomatal closure, which prevents water loss through the transpirational pathway. Drought-induced accumulation of abscisic acid (ABA) promotes a cascade of reactions resulting in stomatal closure to prevent water loss (Daszkowska-Golec & Szarejko 2013). Therefore, ABA-mediated stomatal movement represents an attractive target in efforts to improve drought tolerance in crops. Abscisic acid inhibits the activity of plasma membrane proton (H^+)-ATPases, resulting in stomatal closure under drought stress (Xue et al. 2018). In contrast, constitutive activation of plasma membrane H^+-ATPase 1 (AHA1) prevents ABA-mediated stomatal closure (Merlot *et al*. 2007). These results suggest that downregulation of plasma membrane H^+-ATPase is required for ABA-induced stomatal closure. In *Arabidopsis*, the gene encoding OPEN STOMATA 2 (OST2), previously named AHA1, has been successfully modified using the tissue-specific Cas9 cassette guided by a tru-gRNA (Osakabe *et al*. 2016). A 1-bp insertion at the target site of the OST2-CRISPR2 homozygous mutant causes a frameshift and introduces a stop codon to create a dominant-negative *OST2* mutation, which enhances ABA-induced stomatal closure resulting in a lower rate of transpirational water loss compared with that of the wild-type and T-DNA insertion mutant (Osakabe *et al*. 2016).

MicroRNAs (miRNAs) have been recognised as potential targets of genetic engineering to improve abiotic stress tolerance in crops. Small non-coding endogenous RNA molecules, MiRNAs regulate the expression of cognate target genes by directing mRNA cleavage, translational repression, chromatin remodeling or DNA methylation (Shriram *et al*. 2016). Depending on their target genes, transgenic plants with altered miRNA expression showed either enhanced tolerance or sensitivity against different abiotic stresses (Zhang 2015). Similarly, the null mutant, or so-called miR169a gene knockout, a negative factor of drought tolerance controlled by the ABA-dependent pathway (Li *et al*. 2008; Ni *et al*. 2013) generated using the dual-sgRNA CRISPR/Cas9 system, exhibits drought tolerance compared to wild-type plants (Zhao et al. 2016), suggesting that functional knockout of miRNA using the CRISPR/Cas system is a feasible strategy for miRNA-based crop breeding. In addition, miRNA393 (miR393-overexpressing rices are more sensitive to drought) (Xia *et al*. 2012) and miR408 (*Arabidopsis* miR408 plays a negative role in drought tolerance) (Ma *et al*. 2015) might be good candidates for improving drought tolerance using the CRISPR/Cas system, although miRNAs respond to abiotic stress in genotype-, plant tissue-, stress- and miRNA-dependent manners (Zhang 2015).

CRISPR/Cas9-mediated DNA repair in untranslated regions (UTRs) of target genes has been used to modulate the level of expression of target genes. In maize, the constitutive promoter *GOS2* was inserted into the 5'-UTR of the native *ARGOS8* (auxin-regulated gene involved in organ size) gene or was used to replace the native *ARGOS8* promoter (Shi *et al*. 2017). Maize ARGOS8 is a negative regulator of the ethylene response, and *ARGOS8*-overexpressing maize exhibits increased grain yield under drought conditions (Shi *et al*. 2015). Similarly, ARGOS8 variants generated by the *GOS2* promoter that insert or swap at the CRISPR-RNA target sites due to homologous

Table 1. Application of the CRISPR-based genome editing approach in plants for improvement of abiotic tolerance.

Species	Target gene	Delivery method/main strategy	Improved trait	Reference
Arabidopsis	OPEN STOMATA 2 (OST2)	Agrobacterium-mediated transformation / truncated-gRNAs (tru-gRNAs) in the CRISPR/Cas9 system to produce site-directed modifications.	Drought stress tolerance	Osakabe *et al*. 2016
Arabidopsis	miR169a	Agrobacterium-mediated transformation / dual-sgRNA/Cas9-mediated targeted deletion to create null mutations.	Drought stress tolerance	Zhao *et al*. 2016
Maize	AUXIN-REGULATED GENE INVOLVED IN ORGAN SIZE 8 (ARGOS8)	Biolistic-mediated transformation / CRISPR/Cas9-mediated DNA repair in untranslated regions of target genes to produce overexpression.	Drought stress tolerance	Shi *et al*. 2017
Arabidopsis	*Arabidopsis thaliana* vacuolar H^+-pyrophosphatase (AVP1)	Agrobacterium-mediated transformation / CRISPR/Cas9 activation system to produce overexpression of target gene.	Drought stress tolerance	Park *et al*. 2017
Arabidopsis	ABA-responsive element- binding protein 1 (AREB1)	Agrobacterium-mediated transformation / CRISPR activation system to enhance the expression of target gene.	Drought stress tolerance	Roca Paixão *et al*. 2019
Rice	Rice type-B response regulator (OsRR22)	Agrobacterium-mediated transformation / CRISPR/Cas9 system to generate knockdown plants.	Salinity tolerance	Zhang *et al*. 2019

recombination exhibit a significantly increased level of *ARGOS8* expression, resulting in improved maize grain yield under field conditions of drought stress (Shi *et al*. 2017). In addition, the CRISPR-mediated gene activation system has also been used to enhance transcriptional activation. In *Arabidopsis*, the CRISPR/Cas9 activation system was used to increase the endogenous transcriptional level of *Arabidopsis thaliana* vacuolar H^-–pyrophosphatase (AVP1) by adding the p65 transactivating subunit of NF-kappa B and a heat-shock factor 1 activation domain to the deactivated Cas9 (dCAS9) bound with the herpes simplex virus VP64 (tetramer of VP16) activation domain. The AVP1- activated plants exhibited 2- to 5-fold increases in AVP1 expression, resulting in enhanced drought tolerance (Park *et al*. 2017). A different version of the CRISPR activation system, CRISPR/dCas9 fusion with a histone acetyltransferase, has been introduced to enhance the expression of ABA-responsive element-binding protein 1 (AREB1), a transcription activator of ABRE (ABA-responsive element)-dependent ABA signaling (Roca Paixão *et al*. 2019). AREB1-activated plants exhibit enhanced drought tolerance, higher chlorophyll content and a faster stomatal aperture under a water deficit (Roca Paixão *et al*. 2019). Taken together, genes involved in molecular mechanisms of the ABA-mediated response to drought will be useful gene resources to improve drought tolerance using the CRISPR/Cas system.

CRISPR/CAS SYSTEM-DIRECTED GENOME ENGINEERING TO ENHANCE SALT STRESS TOLERANCE

The beginning of the 21st century was marked by increased salinisation of soil and water through a rising sea level and salt water intrusion. Salinisation areas are increasing at a rate of 10% annually for various reasons, and >50% of all arable land will be salinised by the year 2050 (Jamil *et al*. 2011). Salinity significantly affects plant growth and development via ion toxicity, osmotic stress, nutrient deficiency and oxidative stress (Shrivastava & Kumar 2015). Salinity tolerance is governed by a multitude of physiological and molecular mechanisms that fall into three major categories: osmotic tolerance, ionic tolerance and tissue tolerance (Munns & Tester 2008). Among the number of genes involved in salinity tolerance of crops, the *OsRR22* gene encoding the rice type-B response regulator was knocked down using the CRISPR/

Cas9 system to enhance rice salinity tolerance. Zhang *et al*. (2019) obtained mutant *OsRR22* plants without transferring DNA via segregation in the T1 generation, and suggested that CRISPR/Cas9-induced mutations in the *OsRR22* gene enhance salinity tolerance, but do not significantly influence agronomic traits (plant height, days to 50% flowering, number of tillers per plant, number of grains per panicle, spikelet fertility, 1000-seed weight and yield per plant) under normal field conditions. Although this is only one example of enhancement of salt stress tolerance using the CRISPR/Cas system, there is no doubt that the CRISPR/Cas system is a powerful and promising tool to improve salt stress tolerance in crops.

During the last two decades, genetic engineering to improve salinity tolerance in crops has focused on genes that are involved with antioxidants, ion transport, signal transduction and transcription factors. Among them, genes regulating ion transport via cell membranes make a great contribution in osmotic adjustment (Volkov 2015). Na^+ extrusion out of the cell and detoxification into vacuoles are mediated by the plasma membrane Na^+/H^+ antiporter, salt overly sensitive 1 (SOS1) antiporter and Na^+/H^+ exchanger 1 (NHX1) antiporter, respectively (Hasegawa 2013). Overexpression of *Arabidopsis SOS1* increases salt tolerance of transgenic *Arabidopsis* and tobacco by limiting Na^+ accumulation under salt stress conditions (Shi *et al*. 2003; Yue *et al*. 2012). In addition, transgenic plants by overexpressing *NHK1* exhibit increased capacity of the cell to store Na^+ in the vacuole, resulting in enhanced tolerance to salt stress (Apse *et al*. 1999). Another important regulator of intracellular Na^+ homeostasis is the high-affinity potassium transporter (HKT) family. Similar to SOS1 and NHK1, overexpression of *HvHKT2;1* (subfamily II HKT transporter from *Hordeum vulgare*) in barley leads to increased uptake and translocation of Na^+ to leaves, resulting in enhanced salt stress tolerance (Mian *et al*. 2011). Based on a genome-wide analysis, Na^+ transporters, including SOS1, NHX1 and HKT, have been identified in a number of plant species. Such information can be used to improve salinity tolerance in crops via targeted genome editing using the CRISPR/Cas system.

IMPROVING PLANT TOLERANCE TO THE HIGH TEMPERATURES ASSOCIATED WITH GLOBAL WARMING

The high temperature and heat waves caused by global warming are predicted to have a general negative effect on crop growth and development, which might lead to substantial yield losses and an increased risk for future global food security. It has been suggested that a temperature increase of 3-4°C could cause crop yields to fall by 15-35% in Africa and Asia and by 25-35% in the Middle East (Bita & Gerats 2013). Mechanisms of crop protection under high temperature-induced heat stress have been linked to increased thermo-tolerance of the photosynthetic apparatus because heat stress causes a reversible reduction in photosynthesis by damaging photosystem-II, reducing the efficiency of electron transport and inducing the rapid production and accumulation of reactive oxygen species (ROS) (Wang *et al*. 2018). The heat stress-response signal transduction pathway, involving heat shock transcription factors (HSFs) and heat shock proteins (HSPs), is associated with the accumulation of ROS (Awasthi *et al*. 2015). Therefore, tolerance to heat stress can be enhanced by increasing the capacity of plants to scavenge ROS (Parmar *et al*. 2017), indicating that thermo-tolerance should be improved by increasing the antioxidant capacity of crops. In plants, a complex antioxidant defence system, including enzymatic and nonenzymatic antioxidants, exists to control redox homeostasis (You & Chan 2015). Superoxide dismutase, ascorbate peroxidase (APX), catalase (CAT), glutathione peroxidase, monodehydroascorbate reductase, dehydroascorbate reductase, glutathione reductase, glutathione S-transferase (GST) and peroxiredoxin are crucial enzymes involved in ROS detoxification (You & Chan 2015). It has been suggested that heat stress-tolerant cultivars contain antioxidant enzymes with higher activities, the group of such enzymes including GST, APX and CAT (Asthir 2015). In addition, transgenic *Arabidopsis* overexpressing the broccoli *CAT* gene and the Chinese cabbage *APX* gene exhibits enhanced heat tolerance (Chiang *et al*. 2014, 2015). The antioxidant defence system is correlated with heat tolerance and other stress-resistance mechanisms of plants (Nguyen *et al*. 2018). These findings indicate that antioxidant enzymes should be targets in using a genome-editing approach to generate multi-stress– resistant plants.

HSPs are classified into five major families, viz., HSP100/ClpB, HSP90/HtpG, Hsp70/DnaK, HSP60/GroEL and small HSPs, according to their molecular weight, and are divided into two groups, viz., ATP-dependent (foldase group) or independent (holdase group) chaperones (Askari-Khorasgani & Pessarakli 2019). HSPs are known as molecular chaperones involved in the transport, folding, assembly and degradation of other proteins (Xu *et al*. 2011). HSP70 is the most abundant in most organisms and acts as a molecular chaperone for cellular survival of heat stress and other types of environmental stresses (Kregel 2002). Overexpression of *HSP70* genes confers thermo-tolerance and enhances resistance to environmental stresses (Wang *et al*. 2004, 2016; Zhao *et al*. 2019). In addition, overexpression of *HSP40*, also known as the DnaJ protein, enhances thermo-tolerance of transgenic tomatoes and *Arabidopsis* (Li *et al*. 2007; Wang *et al*. 2019), indicating that HSPs play an important role in helping to protect plants from high temperature damage. The expression of *HSPs* in response to heat stress is attributed to the conserved heat stress element that serves as the HSF-binding site (Asthir 2015). Under diverse stress conditions, HSFA1d,

HSFA2 and HSFA3 have been suggested to be key players for the expression of *APX*, which performs an important role in the acquisition of thermo-tolerance (Shi *et al*. 2001; Panchuk *et al*. 2002; Jung *et al*. 2013). These results indicate that the HSF-dependent network has important value for improving thermo-tolerance in crops.

CONCLUSIONS

Although CRISPR/Cas-mediated genome editing has become a versatile tool for rapid high-fidelity modification of endogenous genes, only a few studies on applications of CRISPR/Cas-mediated genome editing to improve abiotic stress-tolerance in crops have been conducted compared to the number of studies on model plants (Table 1). The bottlenecks for genome-edited crops are the discovery of target genes, effective delivery of CRISPR machinery to the right cells and regeneration of various crops. Particular attention to abiotic stress response genes and abiotic stress-induced transcriptional networks is required to address the issue of target discovery. In addition, comparative genome-wide analysis will provide a solid foundation for further discovery of the potential target genes in crops. As CRISPR/Cas-mediated plant genome editing still faces challenges, deciphering the regulatory mechanisms of abiotic stress tolerance in model plants by genomic approaches will help to deploy this technology to improve various crops.

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REZIME

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Uređivanje genoma zasnovano na CRISPR/Cas radi poboljšanja tolerancije na abiotski stres kod biljaka

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Klimatske promene utiču na poljoprivredu na više načina, kroz menjanje distribucije vode, dnevnih temperatura i saliniteta. U skladu sa tim, neophodne su inovacije u gajenju biljaka i genetičko inženjerstvo koji imaju za cilj da povećaju toleranciju na biotički stres kako bi se izbeglo smanjenje prinosa izazvano klimatskim promena u 21.veku. Poslednjih nekoliko godina pažnju privlači upotreba CRISPR/Cas sistema za editovanje genoma, kao moćnog sredstva koje može da proizvede nasledne mutacije. Do sada, samo nekoliko studija je pokazalo povećanje tolerancije na abiotički stres pomoću CRISPR/Cas, ali su one jasno sugerisale njegovu efikasnost u budućoj primeni u molekularnom uzgoju, sa ciljem povećanja tolerancije na abiotički stres. Stoga, u ovom revijalnom radu je predstavljena primena CRISPR/Cas sistema za povećanje tolerancije na abiotički stres. Iako efikasnost prilagođavanja i pronalaženja meta za biljne CRISPR/Cas sisteme zahteva dalja poboljšanja, CRISPR/Cas sistemi će predstavljati ključni način za postizanje sigurnosti hrane za vreme klimatskih promena širom sveta.

Ključne reči: CRISPR/Cas sistem, klimatske promene, suša, so, visoka temperatura