RESEARCH ARTICLE

SABRAO Journal of Breeding and Genetics
57 (4) 1528-1534, 2025
http://doi.org/10.54910/sabrao2025.57.4.18
http://sabraojournal.org/
pISSN 1029-7073; eISSN 2224-8978

MANAGEMENT OF DROUGHT, SALT, AND EXTREME TEMPERATURE TOLERANCE IN MAIZE (*ZEA MAYS* L.) USING CRISPR-CAS SYSTEMS

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SUMMARY

The agricultural industry has increasing threats from innumerable negative factors like drought, soil salinity, and the extreme temperatures, as the most noticeable worldwide. These constraints challenge the breeders to either improve existing cultivars or develop new cultivars of crop plants. With advanced biotechnology, the CRISPR-Cas (clustered regularly interspaced short palindromic repeats and associated Cas proteins) system is an effective tool for engineering certain features in various crops, including cotton, maize, soybean, wheat, and barley. Several agriculturally important traits have already gained improvements by using these tools; particularly, yield losses have reached reductions due to enhanced abiotic and biotic stress tolerance with improved quality, and the shelf life of fruits and vegetables is being prolonged. However, for maintaining global food security, the related research community should take measures toward searching for and developing new methods of agricultural advancements. Therefore, in the following review article, we emphasized on the effectiveness of applying the CRISPR/Cas system for improving drought, salt, cold, and heat tolerance in maize (*Zea mays* L.).

Keywords: Maize (*Z. mays* L.), drought, salinity, cold, heat tolerance, gene, genome editing, endonuclease, CRISPR/Cas9

Communicating Editor: Dr. Anita Restu Puji Raharjeng

Manuscript received: September 28, 2024; Accepted: March 05, 2025.

Citation: Kamalova LK, Mirzakhmedov MK, Ayubov MS, Yusupov AN, Mamajonov BO, Obidov NS, Bashirxonov ZH, Murodov AA, Buriev ZT, Abdurakhmonov IY (2025). Management of drought, salt, and extreme temperature tolerance in maize (*Zea mays* L.) using CRISPR-Cas systems. *SABRAO J. Breed. Genet.* 57(4): 1528-1534. http://doi.org/10.54910/sabrao2025.57.4.18.

Key findings: Uzbekistan is a region designated with high aridity, salinity, drought, and water scarcity risks. The CRISPR/Cas9 implementation in plant breeding has emerged as effective for broadening the horizons and managing crop problems with a new look. The CRISPR/Cas9 method was evidently highly beneficial in improving salt, drought, and extreme temperature tolerance in maize (*Z. mays* L.).

INTRODUCTION

Maize (*Zea mays* L.) plays a crucial role in maintaining the global food security. However, its present cultivation is facing several natural constraints, such as drought, saline soils, and extreme temperatures (Kim and Lee, 2023). The necessity of providing the growing population with a nutritious and affordable diet poses a challenge for scientists worldwide. Improving existing genotypes and developing new varieties of economically valuable crops, such as maize, is one of the most imperative goals of modern biotechnology. Currently, in the genetic engineering tools, the CRISPR/Cas system has become very popular. Initially, this mechanism, as discovered by Emmanuelle Charpentier and Jennifer Doudna, used bacteria as a defense mechanism against foreign genetic material (Zhou and Simonian, 2024).

CRISPRs are specific repeated sequences with short spacers, which are formed from nucleotide sequences of phages that invade a bacteria’s cell. Subsequently, when the phage invades the cell again, the transcription of a single guide RNA happens, directing the Cas endonuclease complex to target the invading genetic material. This discovery completely altered the available molecular mechanisms for scientific research in various fields of biotechnology, like medicine, pharmacy, cosmetology, and agriculture. The sequence specificity of this natural immune phenomenon has now been applicable for genetic manipulation in different cells, tissues, and organisms (Feng *et al*., 2022). Numerous studies authenticated the effectiveness of the said technology in genetic engineering used for crop tolerance to various biotic and abiotic stresses. In cereal crops, such technology’s use has already been successful in rice, wheat, and maize (Ahmar *et al*., 2024).

Apart from developing stress-resilient maize cultivars, CRISPR has also been successful in its application for enhancing maize grain yield by editing *CLE* genes (Doll *et al*., 2019). Consequently, it plays an influential role in plant development, with the creation of the world’s first aromatic maize via the inactivation of two aldehyde dehydrogenase genes (Wang *et al.*, 2021). Moreover, through CRISPR/Cas9 editing of the gibberellins oxidase *Gibberellin-Oxidase20-3* gene in maize, the geneticists were able to obtain semi-dwarf maize plants resistant to lodging (Zhang *et al.*, 2020). Another successful implementation of the CRISPR system has reports on developing both single and multiple knockouts in the maize genome (Doll *et al.*, 2019). Past findings have focused more on the theoretical mechanisms underlying plant response to various stresses. However, this presented review aimed to immerse itself deeply into past studies regarding the implementation of the CRISPR/Cas technique in improving the abiotic stress resistance in maize.

CRISPR-mediated improvement in maize salt tolerance

Soil salinization is one of the global problems causing damage to arable soils and crops worldwide. The natural processes contribute to the worsening of this problem; however, human activities also exacerbate this environmental disaster. Soil salinity negatively affects plant growth and development, reduces root growth, and eventually decreases crop yield and quality. Genome-wide association analysis is a perspective technique used for searching genes that play a vital role in plant response to salt tolerance. By assessing 348 maize inbred lines, GWAS helped identify 83 QTLs responsible for salt tolerance, with 12 candidate genes found responsive to salt stress conditions. Functional characterization of three maize genes by CRISPR/Cas9 revealed the overexpression of *ZmCS3* led to higher salt tolerance and the overexpression of *ZmCYP93D1* to salt sensitivity, while knockout of *ZmCYP709B2* enhanced salt tolerance (Liang *et al*., 2021).

The sugar efflux transporter *SWEET* gene family has several functions in crop plants. In studying its role in maize stress response, the use of the CRISPR/Cas9 tool helped develop *ZmSWEET1b* knockout lines. By treating with salt, knockout plants showed higher levels of wilt, and the transcriptional analysis revealed the genes responsible for Na^+ efflux found in roots to the rhizosphere decreased, proving this gene could be a negative regulator of maize salt tolerance (Wu *et al*., 2023). By investigating the transcription factors' role in plant salinity response, it has been notable that the *ZmbHLH32* transcription factor, binding to the *ZmIAA9* gene related to the Auz/*IAA* gene family, was capable of activating the latter. Thus, the previous study disclosed that the overexpression of *ZmbHLH32* led to an enhanced salt tolerance in maize, while its CRISPR/Cas9 knockout led to higher sensitivity (Yan *et al*., 2023).

In plants' physiological response to salinity, the sodium and potassium ions (Na^+ and K^+) play a remarkable role; therefore, in a proper salt tolerance, the maintenance of their appropriate ratio is crucial. The QTL mapping used identified the gene *Zea mays* Na^+ *Content1* (ZmNC1), and applying the CRISPR/Cas9 technology helped develop *ZmNC1* knock out lines of maize. Their results showed mutant lines had increased Na^+ accumulation levels and high salt sensitivity, proving that this gene plays an essential role in the regulation of Na^+ content in maize (Zhang *et al.*, 2018). Furthermore, the *ZmHKT1 (Zea Mays High-affinity* K^+ *Transporter 1)* loss-of-function developed through CRISPR/Cas9 revealed that sodium ions concentration enhanced in mutant lines demonstrating that the *ZmHKT1* gene promotes plant salt tolerance by reducing the Na^+ in maize leaves (Zhang *et al.*, 2018).

CRISPR/Cas9 knockout of high-affinity K^+ transporter in maize (*ZmHKT2*), plays an eminent role in transporting K^+ and maintaining K^+/Na^+ homeostasis, a negative regulator of maize salt tolerance, where the mutant plants appeared larger than wild-type plants in extreme salinity conditions (Cao *et al*., 2019). The salt overly sensitive (*SOS*) pathway is crucial in crop plants under salinity conditions. When studying the mechanism, CRISPR/Cas9 has knocked out the two maize synthase kinase genes (*ZmSK3* and *ZmSK4*) in regulating the SOS pathway. The mutant plants exhibited increased sensitivity to salt stress. Moreover, past findings enunciated that *ZmSOS3* and *ZmSOS3-like calcium-binding 8* (*ZmSCaBP8*) participate in maintaining Na^+/K^+ homeostasis under salt stress condition (Li *et al*., 2023a). The *HAK* family genes were evidently essential for plant salt tolerance, as these genes exclude the Na^+ ions from shoots. The loss of the *ZmHAK4* gene function led to an increase in the content of Na^+ ions and decreased salt tolerance in maize (Zhang *et al.*, 2019).

CRISPR-mediated improvement in maize drought tolerance

Drought stress is an instigating environmental factor caused by water shortage in the soil; it naturally and tremendously affects plant development and yield. Plants have acquired several adaptation techniques comprising variations occurring in abscisic acid, ethylene, and other phytohormone signaling pathways. It is factual knowledge that plant phytohormones are key molecules in plant development and response to environmental stress conditions. Among others, gibberellins play an essential role in plant development, with reports of it supporting plant response to abiotic stress conditions. The CRISPR/Cas9 system has been operational in the modification of the maize *auxin-regulated gene involved in organ size* (*ARGOS*). This gene family negatively regulates the ethylene signaling pathway and enhances the drought tolerance; however, its native expression level is low in maize plants.

Therefore, the GOS2 promoter's insertion into the untranslated region of the *ARGOS8* gene resulted in an improved drought tolerance and increased yield in maize under drought stress conditions (Shi *et al*., 2017).

The identification of drought stress-response genes' expression is crucial. A genome-wide association study served to search the genome for appropriate candidate genes; particularly, in a past study, using the said method took place in 224 maize inbred lines and almost 30,000 candidate genes under drought stress regimes (Liu *et al*., 2020). Overall, 75,573 QTLs achieved their detection in response to drought stress, and among them, the Mendelian randomization analysis revealed 97 main candidate genes, with 51 of them being positive and 46 being negative regulators. According to this research, the *abscisic acid 8′-hydroxylase (abh)* seemed to be one of the negative regulators of maize drought tolerance (Liu *et al*., 2020). The implementation of CRISPR/Cas9 technology to develop *abh2* knock-out maize lines showed that the vitality of mutant lines was significantly higher under water-deficit conditions, proving that this gene plays a negative role in plants' drought stress response (Liu, 2020). These results provide an enormous basis for research toward crop genetic engineering for drought stress tolerance.

A study proceeded to define the *Dof22* gene performance, which encodes DNA-binding with one finger, in maize via CRISPR/Cas9-mediated overexpression of the gene. It revealed mutant maize lines acquired tolerance to drought stress by improved stomatal closure, reduced water loss, and managing the ABA signaling pathways (Cao *et al*., 2019). The maize sulfite oxidase gene has also appeared to be involved in plant abiotic stress response. Its promoter region, induced by drought stress and the highest ABA levels, makes it crucial to study the *ZmSO Pro* promoter region. Previous research stated the 119-bp region of this promoter proved responsible for drought tolerance in mutant plants, making this region a prospective candidate in maize genome editing for drought tolerance (Xu *et al*., 2019). Functional analysis of the maize *tetratricopeptide repeat 1 gene (ZmTPR1)* exhibited that the mentioned gene participated in response to various abiotic stresses, and its expression in drought-resistant maize lines was significantly higher than in drought-sensitive maize plants (Cao *et al*., 2024a, b). Mitogen-activated protein kinase genes were also notably vital for plant response to abiotic stress conditions. Via overexpression and CRISPR knockout of the *ZmMAPK1* gene, showed a positive role in regulating maize seedlings' drought tolerance (Zhu *et al*., 2020).

Transcription factors are essential for plant abiotic stress response, as these factors manage the expression of various genes. For instance, it was apparent that abscisic acid-, stress-, and ripening-induced (ASR) proteins tended to be deeply involved in plant drought stress response. In studying the role of *ASR* genes in maize, the breeders applied the CRISPR/Cas9 system for *ZmASR1* knockout (Yang *et al*., 2024). Their results also revealed that mutant lines had lower reactive oxygen species accumulation and enhanced ABA content and stomatal closure under drought stress conditions. The *MYB* transcription factor family plays a pivotal role in the secondary metabolism and plant stress response. A report has also released that the knockout of the *ZmRL6* gene with relations to *MYB* genes, leads to increased drought sensitivity in maize plants (Zhang *et al*., 2023). The *ZmNST3*, a novel *NAC* transcription factor, incurred knocking out by the CRISPR/Cas9 system to identify its role in maize plants; the study expressed that this gene enhanced the plant drought tolerance and the generation of antioxidant enzymes (Ren *et al*., 2020). A new transcription factor, *ZmEREB24*, related to the AP2/*ERF* transcription factor family, was also noteworthy to be a positive regulator of drought tolerance. However, when knocked out by CRISPR/Cas, the mutant maize seedlings showed higher sensitivity to water-deficit conditions (Ren *et al*., 2024).

Another approach toward plant drought tolerance includes manipulation of those genes critical for plant morphology and development under drought stress conditions. Leaves covered with natural cuticular wax protect them from undesirable environmental factors. CRISPR/Cas9 created loss-of-function of maize *semi-rolled leaf 5* cuticular-wax-related gene causing increased water loss, where studies revealed this gene was crucial in maintaining plant drought stress tolerance via normal cuticular wax production (Pan *et al*., 2020). For a reduced degree of senescence in maize leaves, the scientists knocked out the papain-like cysteine proteases gene *ZmSAG39* via CRISPR/Cas9 (Wang *et al*., 2022a). Mutant maize plants acquired higher seed germination and seedling survival rates, reduced reactive oxygen species content, and lower senescence levels in leaves; and thus, the above-mentioned gene has proven to be a negative regulator in plant drought tolerance (Wang *et al*., 2022b).

CRISPR-mediated improvement in maize tolerance

With global warming, extreme temperatures occur more often than the norm and even intensely much more in some regions. Heat stress can adversely affect the growth and development of maize plants, thus reducing their yield. As maize, along with rice, is one of the chief cereal crops, it is necessary to develop heat-resistant cultivars to maintain global food security.

Plants usually activate the heat-shock genes in heat stress conditions; thus, a heat-shock family gene, *HSP101,* reached accumulation in pollen mother cells, and its overexpression through CRISPR editing showed enhancement of heat tolerance in maize mutant lines (Li *et al*., 2022). Transcriptome analysis of maize lines under heat stress succeeded in identifying the transcription factors that contribute to maize heat stress response (Li *et al*., 2024). Out of the identified genes, heat shock factor *ZmHSF20,* as knocked out via the CRISPR/Cas9, indicated the maize mutant lines showed increased thermotolerance. Moreover, the *Zmhsf20Zmhsf4* double maize mutant provided a decreased tolerance to heat, revealing that the *ZmHSF20* lowers heat tolerance via repressing *ZmHSF4* (Li *et al*., 2024).

Another group of proteins activated by unfavorable environmental conditions appeared in various protein kinases. Calcium-dependent protein kinases (CDPKs) were evident to be crucial in plant stress response, and particularly, it has been noticeable that the maize *ZmCDPK7* gene was a positive regulator of heat tolerance. Additionally, its overexpression leads to the occurrence of lines with higher thermotolerance, while CRISPR/Cas-mediated knockout leads to higher thermosensitivity in maize mutant lines (Zhao *et al*., 2021).

Cold stress negatively impacts photosynthesis and biomass accumulation, leading to reduced yields. The *glucose-6-phosphate dehydrogenase (G6PDH*) is an enzyme responsible for the generation of NADPH; therefore, it is essential for plant stress response. The CRISPR/Cas9 knockout of *ZmG6PDH1* in maize led to hypersensitivity to cold. Particularly, exchanges in redox balances of NADPH, ascorbic acid, and glutathione manifest, resulting in an increased production of reactive oxygen species, which are harmful to crop plants (Li *et al*., 2023b). Several studies regarding cold stress tolerance in rice ensued. However, a scarcity of the studies regarding the application of CRISPR/Cas for maize cold tolerance prevails, though some candidate genes undergo investigation.

CONCLUSIONS AND FUTURE PROSPECTS

In the recent era, with the emergence of various biotechnological advances, modern plant breeding has changed much. The CRISPR/Cas genome editing technique has been proven to be effective, rapid, and precise for improving the important agronomical traits of crops. As climate change challenges global food security, plant breeders are encountering problems in developing tolerant crop cultivars to such environmental stresses as salinity, drought, and extreme temperatures. A considerable research work has been progressing in functional genomics toward the exploration of genes playing an influential role in maize stress response. However, a great deal remains in defining candidate genes and developing stress-tolerant maize genotypes because of a shortage of data in the scope of cold stress tolerance. Aside from the Cas9, emerging recently developed types of CRISPR endonucleases, such as Cas12a, Cas13, and Cas14a, have the plant breeders effectively using them, as each of them has its benefits and drawbacks. Moreover, some limitations in genome editing delivery methods exist. Nowadays, one of the widely used delivery tools is the *Agrobacterium tumefaciens* method, which is also time-consuming and laborious. The regeneration process of maize plants is still a constraint for tissue-culture researchers. Another in the CRISPR/Cas implementation is the international genome editing regulations. For sustainable agriculture to exist in the contemporary world, the establishment of clear regulatory requirements based on scientific achievements is necessary.

ACKNOWLEDGMENTS

The authors acknowledge the Center of Genomics and Bioinformatics research team for helping to collect information to write the manuscript. The article was written using funding from the State Budget given by the Academy of Sciences of the Republic of Uzbekistan.

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