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REVIEW ARTICLE

Genome editing based trait improvement in crops: current perspective, challenges and opportunities

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Abstract

The exponentially increasing population poses a serious threat to global food security. Concurrently, the climate change condition also limits crop productivity by enhancing the effect of biotic and abiotic stressors. The traditional crop improvement programs are not enough to meet the food and nutritional requirements of such a progressive population. Recently, the clustered regularly interspaced short palindromic repeats (CRISPR)/CRISPR-associated protein (Cas) based genome editing tool adopted from bacterial adaptive immune system against invading foreign DNA has demonstrated its tremendous potential in the sector of crop improvement. CRISPR/Cas-mediated targeting can activate, repress, or completely abolish the gene function. CRISPR/Cas-mediated interventions can produce biofortified crops by targeting negative regulators or activating positive regulators for nutrients. Thus, it can address nutritional security concerns. The advancement in CRISPR/Cas-mediated genome editing, encompassing base and prime editing has paved the way to modify an organism's genome in a predictable and precise manner. The use of morphogenetic regulators can omit the problem of tissue culture stages, which is one of the major bottlenecks in plant genome editing. CRISPR/Cas-based genome editing has been performed in many crop plants to induce biotic and abiotic stress tolerance, increase quality and nutritional values, enhance productivity, and prevent post-harvest losses. In this review article, we summarize the progress, challenges opportunities and regulatory landscape of genome editing for the improvement of various traits in crop plants.

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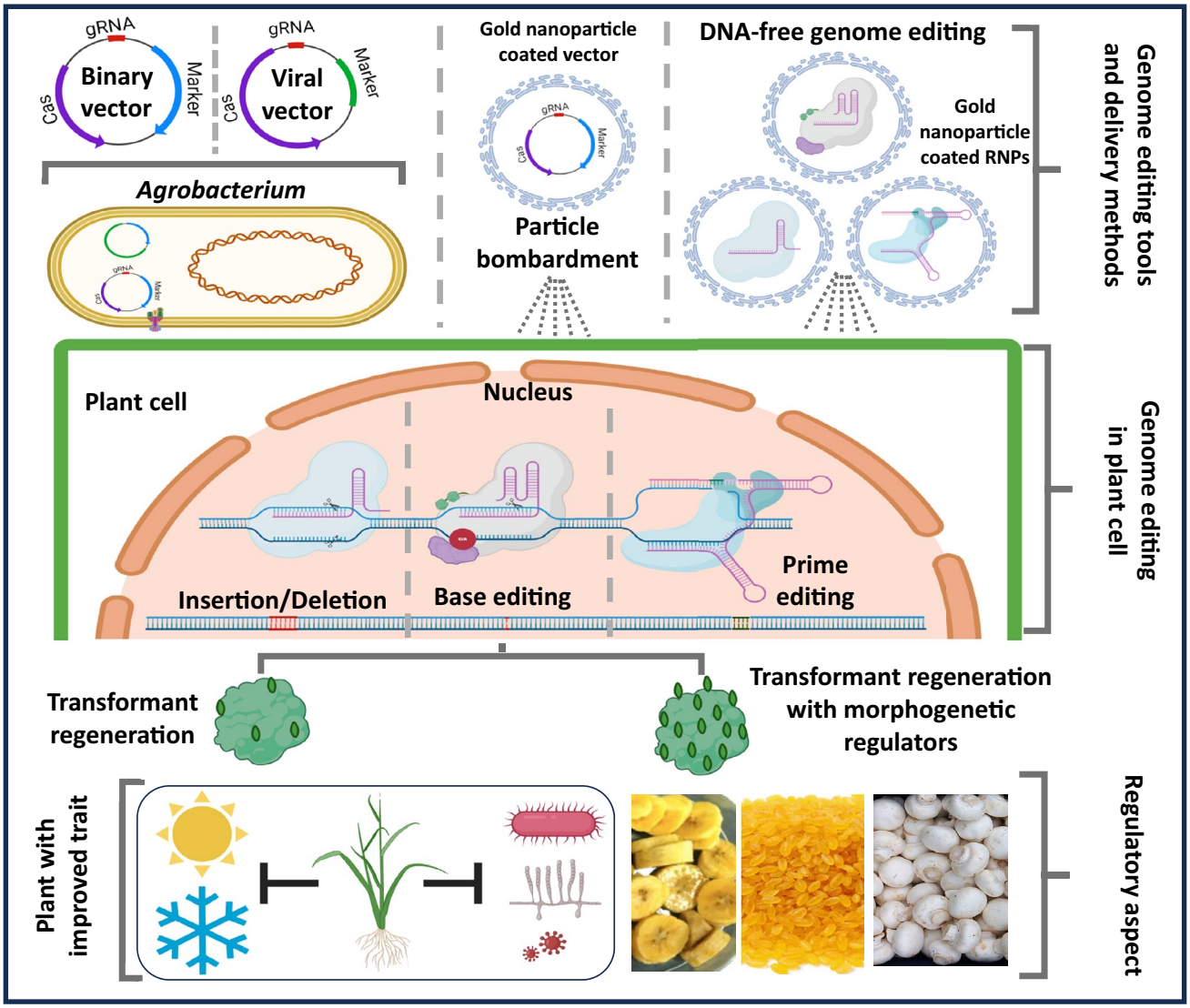
Graphical abstract



Keywords Abiotic and biotic stress · Base editing · CRISPR/Cas · Crop improvement · Genome editing · Prime editing

Introduction

It is estimated that by the year 2050, environment, agriculture, and food systems will face major challenges worldwide [118]. As the global population will reach around 9.7 billion, there will be a huge rise in agricultural products demand compared to 2012. Despite substantial investments in agricultural and technological progress, crop production has not made significant progress over the past thirty years mainly due to declining agricultural lands and an increasing population burden. The annual growth in yields of staple crops worldwide has averaged a little over 1% since the 1990s, a notably slower pace compared to the 1960s [43]. The necessity for an accelerated increase in crop productivity and quality becomes imperative due to factors such as climate change, ever-growing population, depletion of resources, loss of biodiversity and the emergence of plant pests and diseases [43].

Throughout the extensive history of crop domestication, various methods such as conventional plant breeding, mutation breeding, insertion of transgenes, and genome editing have been utilized for the introduction of various traits. Traditional hybridization and mutation-based breeding although considered effective, often require prolonged periods, significant labor input, and also carry unwanted traits due to less precision. The development of transgenic gained rapid

momentum in the past century, emerging as a promising technology for incorporating multiple desirable traits into a variety. However, certain limitations were imposed initially because of the disputes that arose from the safety and ethical considerations of transgenic plants [33]. The clustered regularly interspaced short palindromic repeats (CRISPR)/CRISPR-associated protein (Cas) system (CRISPR/Cas) has become widely acclaimed and is an extensively utilized genome editing tool due to its affordability, adaptability, and precise genetic manipulation [121]. Its successful application has been implemented in numerous economically significant crops. Recent developments in CRISPR/Cas-based editing have directed new avenues for improving relatively complicated traits in various crop plants.

Genome editing assisted by sequence-specific nucleases (SSNs)

The emergence of sequence-specific nucleases (SSNs) facilitates the precise manipulation of a particular gene sequence using genome editing technologies (Fig. 1). The SSNs demonstrated to achieve efficient gene editing involve meganuclease, zinc finger nuclease (ZFN), transcription activator-like effector nuclease (TALEN), and CRISPR/Cas. Genome editing through the CRISPR/Cas system holds the substantial potential to enhance crop genetics and advance molecular breeding, primarily because of its cost-effectiveness, accuracy, and less time-consuming process.

Meganucleases, commonly denoted as endonucleases, interrogate a large site spanning approximately 12–40 base pairs (bp). This unique characteristic renders meganucleases highly effective carriers for all types of vectors, including those associated with plant RNA viruses. Conversely, meganuclease poses challenges when it comes to reengineering in comparison with alternative genome-targeting strategies, as their DNA-binding and catalytic domains are often intricately linked and not easily separable [121]. ZFN, an early genome editing technology developed in the 1990s, enables targeted alterations at specific genomic locus. It encompasses a zinc finger protein that identifies and binds to particular DNA sequences, along with the non-specific DNA-cutting nuclease, *Fok*I. ZFN operates as a dimer, requiring a DNA-binding domain to facilitate attachment to the target, followed by *Fok*I-mediated endonuclease activity for double-strand DNA breakage, initiating the endogenous DNA repair response [71]. Despite its high target binding efficiency, ZFN's limitations include

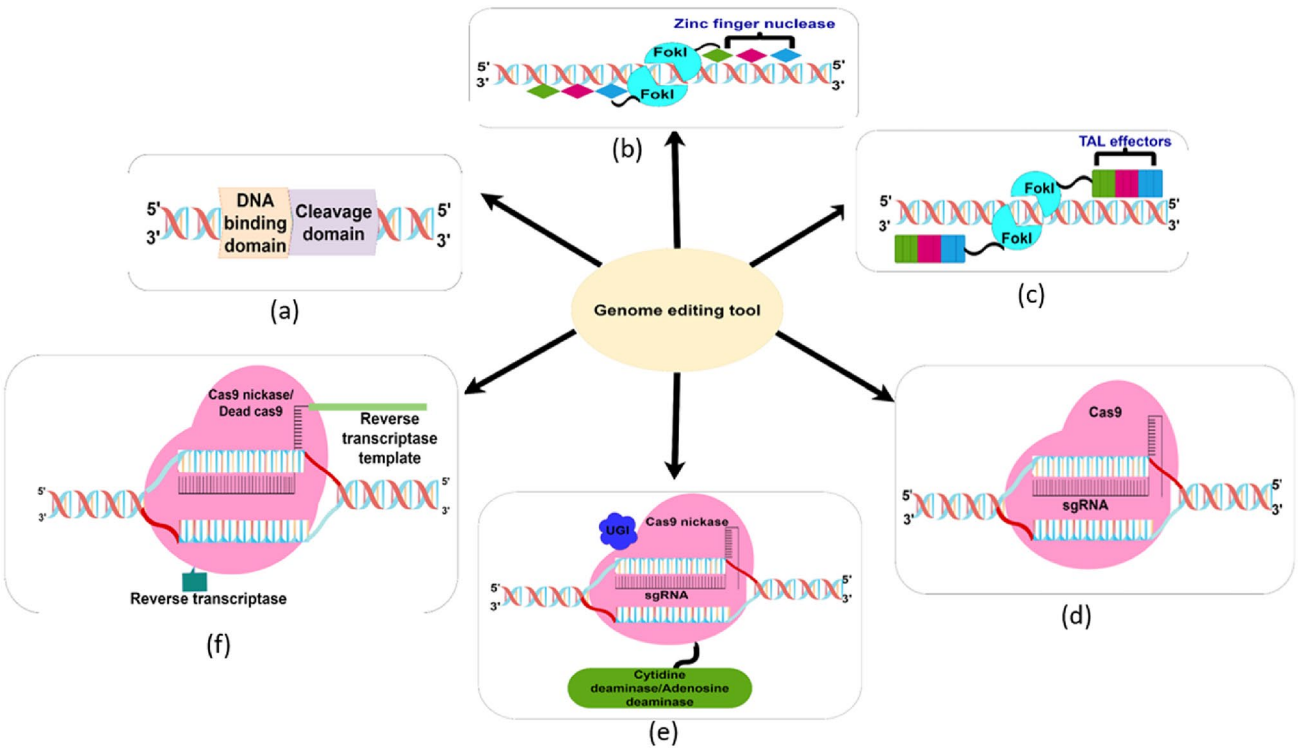


Fig. 1 Overview of different genome editing techniques. **a** Meganucleases or homing endonucleases. **b** Zinc finger nucleases comprise with zinc finger DNA binding domain and *Fok*I endonuclease. **c** TALE effector nucleases involving TAL effectors fused to *Fok*I endonuclease. **d** CRISPR/Cas, adopted from bacterial adaptive immune system. **e** Base editing is a modification of CRISPR/Cas by fusing deaminase to catalytically altered Cas protein. **f** Prime editing- a search and replace tool for genome editing

a constrained zinc finger protein repertoire, a costly design and a restricted pool of recognizable genomic sequences. These factors collectively curtail the efficiency of genome editing, leading to a decline in its use. In 2009, the TALEN emerged as an advancement from ZFN, boasting enhanced design flexibility. TALEN comprises two components, a cleavage domain featuring the restriction endonuclease *Fok*I and a DNA binding domain to recognize specific DNA sequences. The binding domain incorporates repetitive conserved sequences derived from the TALE protein present in the *Xanthomonas* spp. This domain contains 34 highly conserved amino acids, having 12th and 13th variable residues imparting specific recognizing capability. After recruitment on target loci, TALEN forms a dimer to induce double-stranded breaks (DSBs) in the sequence spacer of TALEN. This prompts the DNA repair mechanism to perform gene editing [197]. Unlike ZFN, TALEN's doublets of variable amino acid residues can recognize diverse nucleic bases, simplifying design and facilitating screening. However, implementing the TALEN in plant genome editing is considered technically difficult, resulting in lower editing efficiency and higher costs compared to ZFN.

The CRISPR/Cas system employs a Cas9 endonuclease in conjunction to a complex of RNA consisting of CRISPR RNA (crRNA) and trans-activating CRISPR RNA (tracrRNA) to facilitate precise DNA cleavage at target sites. The Cas9 protein initiates the double-stranded DNA cleavage before a protospacer-adjacent motif (PAM). For instance, NGG is the PAM sequence for *Streptococcus pyogenes* (Sp) Cas9 at locations that align with the crRNA sequence. The DSBs amended by DNA repair processes of cells lead to editing at target loci (Fig. 2).

CRISPR/Cas: a bacterial adaptation against invading foreign DNA

CRISPR is an adaptive defense mechanism found in the majority of described archaea and bacteria. CRISPR/Cas systems have been classified into two main groups based on their components and mechanisms. In class 1 (types I, III, and IV), the process of RNA-guided target cleavage requires a complex of multiple effector proteins. While class 2 (types II, V, and VI) operate with just one RNA-guided endonuclease. For instance, Cas9 belongs to type II and *Prevotella* and *Francisella* 1 (Cpf1) in type V are responsible for carrying out the target cleavage process [113].

CRISPR/Cas-mediated immunity involves three phases [144]. The acquisition of foreign DNA sequences known as protospacer at the CRISPR locus initiates adaptive immunity. The synthesis of Cas proteins and transcription of the CRISPR array leads to the generation of pre-crRNA which is matured in subsequent steps. Then finally Cas protein in conjunction with mature crRNA performs cleavage at the target locus [39]. The absence of PAM near the crRNA target site

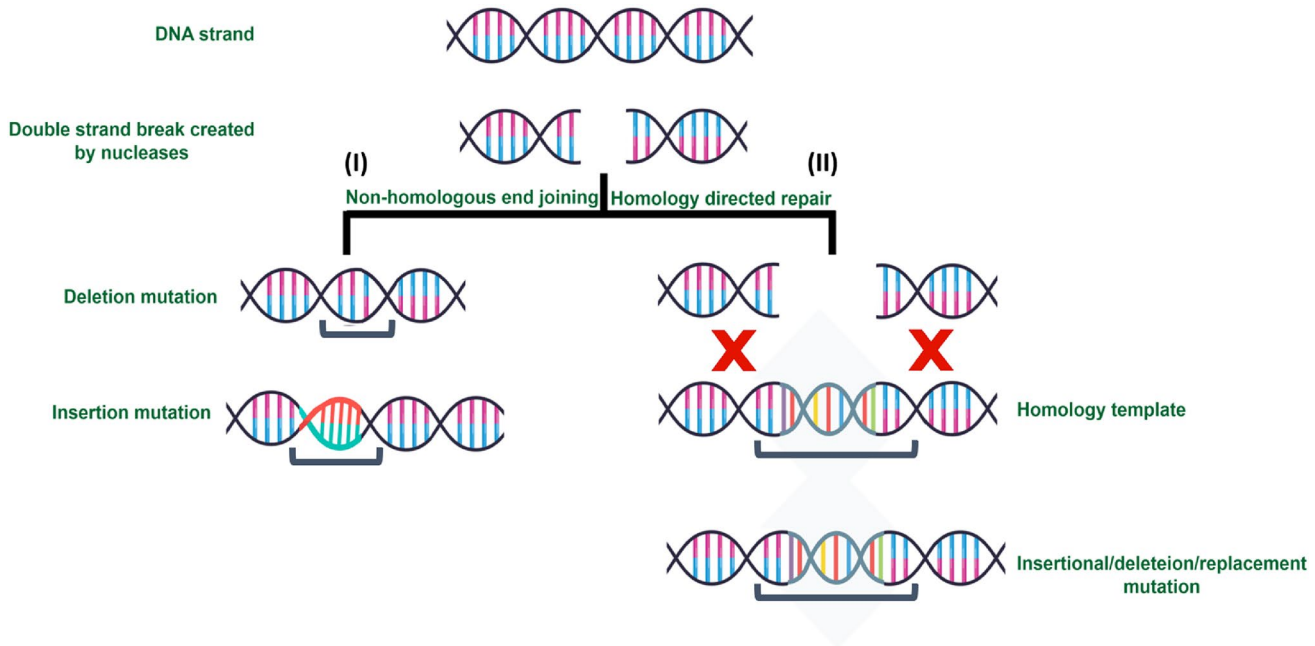


Fig. 2 Major DNA damage repair pathways in plants. **I** Non-homologous end joining (NHEJ) is an error-prone DNA damage repair that introduces insertion or deletion (InDels) randomly, hence employed in gene knockouts. **II** Homology-dependent repair (HDR) pathway which relies on the presence of repair templates for gene knock-in/replacement

in the host genome CRISPR locus protects themselves from the self-cleavage in type I and type II CRISPR systems [67].

Reprogramming of the CRISPR/Cas system for targeted genome editing

Cas endonuclease can be targeted to any genomic loci just by changing the protospacer region specific to the loci of interest. The combination of Cas endonuclease and sgRNA has the remarkable ability to target virtually any genomic location, leading to the creation of DSBs [116]. These DSBs can be repaired via two main natural repair pathways, the less precise non-homologous end-joining (NHEJ) repair or the more accurate homology-directed repair (HDR) pathways [170]. NHEJ often results in gene knockouts, while HDR allows for precise insertion-based modifications of DNA sequences. In higher plants, NHEJ is more commonly observed compared to HDR [163]. The HDR pathway needs a repair template for homologous recombination to resolve the DSBs. HDR-based repairing property presents several opportunities such as introducing precise single-base changes, diversifying localized sequences, creating novel versions of proteins and expediting the evolution of specific proteins to develop cultivars with enhanced abiotic/biotic tolerance.

Crop improvement with CRISPR/Cas-based genome editing

Since CRISPR/Cas-based genome editing offers immense potential to modify genomes in a very efficient and well-predicted manner, it has been employed in many crops to improve abiotic and biotic stress, nutritional and quality-related traits. However, some of the regulatory and ethical concerns limit the worldwide use of genome editing in better exploration and public accountability (Fig. 3). The advancements in genome editing technologies are stepping forward to overcome these stumbling stones. The recent developments involving the utilization of genome editing for various trait improvements are explored in the following sections and summarized in Table 1.

Improving salinity stress tolerance

Salinity stress is a critical abiotic challenge impacting both fertile lands and crop productivity and ranked second in severity [46]. The overuse of chemicals, comprising pesticides and fertilizers is turning the cultivable lands into saline. Notably, the salt stress resilience of rice was enhanced using CRISPR/Cas9 technology to edit the *B-type response regulator 22* (*RR22*) gene encoding a transcription factor pivotal in cytokinin signaling and metabolism. This genetic alteration resulted in improved salt tolerance observed across two successive generations, without discernible differences between the edited and unedited lines [218]. Similarly, introducing *paraquat tolerance-3* mutations (*PQT3*) through CRISPR/Cas9 led to substantial salt tolerance in rice [6]. The potential of *miR535*, a miRNA gene implicated in salt stress response was explored. The CRISPR/Cas9-mediated disruption of *miR535* showed rice plants ability to withstand salinity. Additionally, a 5 bp deletion within the *miR535* coding region emerged as a viable target for elevating salt tolerance in rice [212].

Further progress in salt stress tolerance was achieved through CRISPR/Cas9-mediated modification of various genes. By eliminating the *basic helix-loop-helix 024* (*bHLH024*) gene and enhancing the ion transporter expression including *high-affinity potassium transporter 1* (*HKT1*),

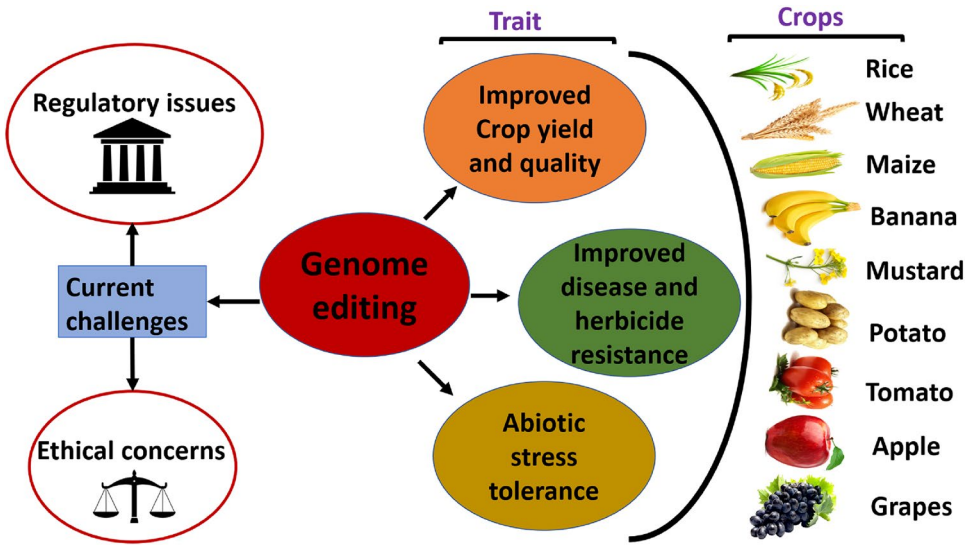


Fig. 3 Applications of genome editing in crop improvement. Current challenges, areas of applications and major crops in which genome editing has been employed for improvement of various traits

Table 1 CRISPR/Cas-mediated genome editing in plants for trait improvement

Plant	Genome editing method	Target locus	Target locus function	Trait improved	References
Oryza sativa	CRISPR/Cas9	B-type response regulator (RR22)	Transcription factor involved in cytokinin signaling and metabolism	Enhanced salt tolerance	[218]
O. sativa	CRISPR/Cas9	*Paraquat tolerance-3* (*PQT3*)	An E3 ubiquitin ligase	Enhanced salt tolerance	[6]
O. sativa	CRISPR/Cas9	*miR535*	A miRNA gene in salt stress response	Enhanced salt tolerance	[212]
O. sativa	CRISPR/Cas9	*Basic helix-loop-helix 024* (*bHLH024*)	A bHLH family transcription factor (TF)	Enhanced salt tolerance	[5]
O. sativa	CRISPR/Cas9	*Related to ABI3 and VP1 2* (*RAV2*)	A TF	Enhanced salt tolerance	[99]
Solanum lycopersicum	CRISPR/Cas9	*Hybrid proline-rich protein 1* (*HyPRP1*)	Plays important role in plant development	Enhanced salt tolerance	[177]
O. sativa	CRISPR/Cas9	*Drought and salt tolerance* (*DST*)	Zinc-finger protein	Enhanced salt tolerance and drought tolerance	[153]
Hordeum vulgare	CRISPR/Cas9	*Inositol 1,3,4-trisphosphate 5/6-kinase (ITPK1)*	Involved in phytic acid biosynthesis	Enhanced salt tolerance	[181]
O. sativa	CRISPR/Cas9	*NAC041*	An NAC family TF	Sensitive to salt stress	[190]
Arabidopsis thaliana	CRISPR/Cas9	*Open stomata 2 (OST2)*	An plasma membrane H^+ATPase AHA1	Improved drought tolerance	[135]
Zea mays	CRISPR/Cas9 based knock-in	*Involved in organ size 2* (*GOS2*)	Auxin-responsive gene	Improved drought tolerance	[220]
A. thaliana	CRISPR/Cas9	*Vacuolar H+-pyrophosphate (AVP1)*	An H^+ translocating phosphatase	Improved drought tolerance	[137]
A. thaliana	CRISPR/dCas9 fusion with Histone acetylase	*Abscisic acid-responsive (ABA) element binding gene 1 (AREB1)*	ABA signaling gene	Improved drought tolerance	[149]
A. thaliana	CRISPR/Cas9	*Trehalase 1*	Trehalose biosynthetic gene involved in abiotic/biotic stress	Improved drought tolerance	[130]
O. sativa	CRISPR/Cas9	*Enhanced response to the ABA1 (ERA1)*	Involved in ABA signaling	Improved drought tolerance	[132]
O. sativa	CRISPR/Cas9	*Semi-rolled leaf 1 (SRL1), SRL2, ERA1*	SRL1 is a glycosylphosphatidylinositol-anchored protein and modulates rice leaf rolling	Improved drought tolerance	[94]
O. sativa	CRISPR/Cas9	*Pyrabactin resistance-like 9* (*PYL9*)	ABA receptor	Enhanced yield and drought tolerance	[179]
O. sativa	CRISPR/Cas9	*OsOREB1* (an ABRE binding TF), *OsRab16b*, *OsRab21*, *OsbZIP23*, *OsLEA3, OsSLAC1*, and *OsSLAC7*	Transcription factors	Improved drought tolerance	[104]
Cicer arietinum	CRISPR/Cas9	*Reveille 7* (*RVE7*) and *4-coumarate ligase* (*4CL*)	RVE7 is an MYB TF and 4CL is a phenylpropanoid metabolic enzyme	Improved drought tolerance	[13]
A. thaliana	CRISPR/Cas9 based knock-in	*miR169a*	An miRNA specific to proteins involved in biotic/abiotic stresses	Improved drought tolerance	[161]

Table 1 (continued)

Plant	Genome editing method	Target locus	Target locus function	Trait improved	References
S. lycopersicum	CRISPR/Cas9	*Gibberellin insensitive dwarf 1* (*GID1*), *lateral organ boundaries domain 40* (*LBD40*), and *mitogen activating protein kinase 3* (*MAPK3*)	GID1 is a gibberellin receptor, LBD is TF and MAPK3 is involved in signaling cascades	Improved drought tolerance	[103, 183]
Z. mays	CRISPR/Cas9	*Gibberellic acid 20 oxidase 3* (*GA20ox3*)	A gibberellin biosynthetic enzyme	Semi-dwarf maize resistant to drought stress	[98]
S. lycopersicum	CRISPR/Cas9	*Non-expressor of pathogenesis-related 1* (*NPR1*)	A pathogenesis-related (PR) gene	Improved drought tolerance	[89]
Triticum aestivum	CRISPR/Cas9	*Ethylene response factor 3* (*ERF3*) and *dehydration-responsive element binding protein 2* (*DREB2*)	ERF3 is an ethylene receptor and DREB2 is a TF	Improved drought tolerance	[70]
Triticum aestivum	CRISPR/Cas9	*3′(2′),5′-bisphosphate nucleotidase* (*Sal1*)	Hydrolase enzyme coding gene	Improved drought tolerance	[1]
Gossypium hirustum	CRISPR/Cas9	*Homeobox 12* (*HB12*)	A homeodomain-containing proten	Improved drought tolerance	[48]
Brassica napus	CRISPR/Cas9	*Repressor of GA1*-3 (*RGA*)	A repressor of gibberellin signaling	Improved drought tolerance	[199]
S. lycopersicum	CRISPR/Cas9	*MAPK3* and *agamous-like 6* (*AGL6*)	*AGL6* is a developmental regulator encoding gene	Enhanced heat stress tolerance	[22]
S. lycopersicum	CRISPR/Cas9	*Brassinazole-resistant 1* (*BZR1*)	Brassinosteroid receptor	Enhanced heat stress tolerance	[208]
Z. mays	CRISPR/Cas9	*Heat-stress-sensitive albino 1* (*HSA1*)	Involved in chloroplast development and abiotic stress resposne	Enhanced heat stress tolerance	[143]
Lactuca sativa	CRISPR/Cas9	*9-cis-epoxycarotenoid dioxygenase* (*NCED4*)	ABA biosynthetic gene	Enhanced heat stress tolerance	[19]
O. sativa	CRISPR/Cas9	*Epidermal patterning factor* (*EPF1*)	Involved in Stomatal patterning	Increases temperature sensitivity	[145]
S. lycopersicum	CRISPR/Cas9	*Calcium-dependent protein kinase 2* (*CPK2*)	Calcium signaling protein	Increased thermosensitivity	[53]
O. sativa	CRISPR/Cas9	*TIFY1a* and *TIFY1b*	TIFY gene family proteins	Increased yield and drought tolerance	[58]
O. sativa	CRISPR/Cas9	*proline-rich protein 1* (*PRP1*)	Encodes class of intrinsically unstructured proteins (IUP) several short proline-rich repeats	Improved cold tolerance	[125]
O. sativa	CRISPR/Cas9	*MYB30*	A TF	Improved cold tolerance	[108]
O. sativa	CRISPR/Cas9	*Rice Gα* (*RGA1*), *GS3*, *dense and erect panicle 1* (*DEP1*), and *putative extra-large G protein 4* (*PXLG4*)	G proteins involved in signaling	Improved cold tolerance	[36]
S. lycopersicum	CRISPR/Cas9	*C-repeat binding factor 1* (*CBF1*)	A bHLH TF	Improved cold tolerance	[92]
A. thaliana	CRISPR/Cas9	5-*oxoprolinase* 1 (*OXP1*)	Involved in glutathione degradation pathway	Improved heavy metal stress tolerance against cadmium	[14]
O. sativa	CRISPR/Cas9	*Natural resistance-associated macrophage protein 1* (*NRAMP1*)	Transporter for ions uptake	Improved heavy metal stress tolerance against cadmium and lead	[35]

Table 1 (continued)

Plant	Genome editing method	Target locus	Target locus function	Trait improved	References
O. sativa	CRISPR/Cas9	*Arsenite-responsive MYB1 (ARM1)*	R2R3 MYB transcription factor controlling arsenite uptake	Improved heavy metal stress tolerance against arsenic	[184]
O. sativa	CRISPR/Cas9	*NRAMP5*	Transporter for ions uptake	Improved heavy metal stress tolerance against cadmium	[172]
O. sativa	CRISPR/Cas9	*HAK1* (Cs^+-permeable K^+ transporter)	Rice potassium transporter	Improved heavy metal stress tolerance against cesium	[128]
O. sativa	CRISPR/Cas9	*Acetolactate synthase (ALS)*	Enzyme involved in branched-chain amino acid biosynthesis	Induced herbicide tolerance	[175]
Z. mays	CRISPR/Cas9	*ALS*	–	Induced herbicide tolerance	[166]
Citrullus lanatus	CRISPR/Cas9	*ALS*	–	Induced herbicide tolerance	[76, 193]
O. sativa	CRISPR/Cas9	*5-enolpyruvylshikimate-3-phosphate synthase (EPSPS)*	Enzyme involved in aromatic amino acid biosynthesis	Induced herbicide tolerance	[155]
Linum usitatissimum	CRISPR/Cas9	*EPSPS*	–	Induced herbicide tolerance	[85]
S. lycopersicum	CRISPR/Cas9	*ALS, EPSPS, PDS*	Phytoene desaturase is an important enzyme in carotenoid biosynthesis	Induced herbicide tolerance	[206]
T. aestivum	CRISPR/Cas9	*mildew resistance locus (Mlo)*	Acts as powdery mildew susceptibility factor	Induced powdery mildew resistance	[158]
S. lycopersicum	CRISPR/Cas9	*mildew resistance locus (Mlo)*	–	Induced powdery mildew resistance	[185]
T. aestivum	CRISPR/Cas9	*enhanced disease resistance 1* (*EDR1*)	Involved in regulation of MAPK activity	Induced powdery mildew resistance	[217]
S. lycopersicum	CRISPR/Cas9	*powdery mildew resistance 4* (*PMR4*)	Callose synthase gene	Induced powdery mildew resistance	[152]
O. sativa	CRISPR/Cas9	*ethylene response factor 922*	Involved in thylene signaling	Induced rice blast resistance	[191]
O. sativa	CRISPR/Cas9	*subunit of the exocyst complex 3A* (*SEC3A*)	Exocyst complex **gene** family member	Induced rice blast resistance	[110]
S. lycopersicum	CRISPR/Cas9	*miR482b* and *miR482c*	Negative regulator of disease resistance genes	Induced resistance against tomato late blight	[52]
S. lycopersicum	CRISPR/Cas9	*Pectate lyase*	Role in fruit softening	Gray mold resistance	[162]
Cucumis sativus	CRISPR/Cas9	*Eukaryotic translation initiation factor 4E* (*eIF4E*)	Viral resistance	Induced resistance against ipomoviruses	[28]
A. thaliana	CRISPR/Cas9	*eIF4E*	Viral resistance	Induced resistance against potyvirusess	[141]
O. sativa	CRISPR/Cas9	*eIF4G*	Viral resistance	Induced resistance against rice tungro spherical virus (RTSV)	[111]
N. benthamiana	CRISPR/Cas9	Coat protein (CP), intergenic region (IR) and replication protein (Rep)	Components of viral replication machinery and its assembly	Induced resistance against Tomato yellow leaf curl virus (TYLCV)	[7]
S. lycopersicum	CRISPR/Cas9	*Dicer-like 2* (*DCL2*)	Role in DNA methylation, RNA interfernce	Induced resistance against tomato mosaic virus (ToMV)	[187]
O. sativa	CRISPR/Cas9	Sucrose transporter *SWEET13*	Sucrose transporter	Induced resistance against bacterial blight	[221]

Table 1 (continued)

Plant	Genome editing method	Target locus	Target locus function	Trait improved	References
Dunken grapefruit	CRISPR/Cas9	*Lateral organ boundary 1* (*LOB1*)	Interact with TALE effector proteins	Induced resistance against canker disease	[63]
Citrus sinensis	CRISPR/Cas9	*LOB1* promoter	Interact with TALE effector proteins	Induced resistance against canker disease	[139]
S. lycopersicum	CRISPR/Cas9	*Jasmonate-ZIM domain protein 2* (*JAZ2*)	Enhance defense reponse against *P. syringae*	Induced resistance against bacterial leaf spot	[134]
Musa acuminata	CRISPR/Cas9	*downy mildew resistance 6* (*DMR6*)	Encodes enzyme which is susceptible to bacterial infection	Induced resistance against *Xanthomonas* wilt	[178]
S. lycopersicum	CRISPR/Cas9	*DMR6*	Encodes enzyme which is susceptible to bacterial infection	Induced resistance against bacteria, oomycetes and fungi	[174]
Ocimum basilicum	CRISPR/Cas9	*DMR6*	Encodes enzyme which is susceptible to bacterial infection	Induced resistance against downy mildew	[80]
Malus domestica	CRISPR/Cas9	*DspA/E-interacting proteins from Malus* (*DIPM*)	*DIPM* interaction with *DspA/E* effector protein leads to fire blight susceptibilty	Induced resistance against fire blight disease	[115]
O. sativa	CRISPR/Cas9	*Tryptamine 5-hydroxylase*	Role in melatonin synthesis	Induced resistance against plant hopper and stem borer	[106]
Glycine max	CRISPR/Cas9	*Hap3*	Increase isoflavone content	Induced resistance against common cutworm	[82]
S. lycopersicum	CRISPR/Cas9	*Alcobaca* (*ALC*)	Increases shelf life and delay fruit ripening	Improved storage properties	[79]
S. lycopersicum	CRISPR/Cas9	*pectate lyase*	Role in fruit softening	Improved shelf life	[211]
M. acuminata	CRISPR/Cas9	*1-aminocyclopropane-1-carboxylate oxidase1* (*ACO1*)	Role in conversion of *1-aminocyclopropane-1-carboxylic acid* (*ACC*) into ethylene	Delayed ripening of banana	[54]
Solanum melongena	CRISPR/Cas9	*polyphenol oxidase2* (*PPO2*)	Causes oxidative browning	Improved shelf life	[73]
L. sativa	CRISPR/Cas9	*squamosa promoter binding protein-like 13* (*SPL13*)	Role in inflorescence development	Increased biomass and leaf density	[18]
S. lycopersicum	CRISPR/Cas9	*PSY1*, *MYB12*, and *SGR1*	PSY1- carotenoid biosynthestic enzyme, MYB12- a TF,SGR1-regulator of chlorophill degradation	Improved fruit pigmentation	[205]
O. sativa	CRISPR/Cas9	*GS3*,	Positive regulator of grain weight and size	Elevated grain length	[160, 214]
S. lycopersicum	CRISPR/Cas9	*OVATE*, *CLAVATA* (*CLV*), *WUSCHEL* (*WUS*), and *excessive number of floral organs* (*ENO*)	Role in plant growth and development	Improves size and shape of fruit	[213, 223]
T. aestivum	CRISPR/Cas9	*Waxy (Wx)*	Amylose synthesis	Low amylose content	[200]
O. sativa	CRISPR/Cas9	*Amino acid permease 6* (*AAP6*) and *AAP10*	Positive regulator of grain growth and production	Increase eating and cooking quality	[194]

Table 1 (continued)

Plant	Genome editing method	Target locus	Target locus function	Trait improved	References
O. sativa	CRISPR/Cas9	*Betaine aldehyde dehydrogenase 2 (BADH2)*	Play role in the synthesis of aromatic compound (2-acetyl-1-pyrroline)	Results in aromatic rice variant	[10]
O. sativa	CRISPR/Cas9	*Phytoene synthase* (*PSY*)	Role in carotenoid and chlorophyll biosynthesis pathway	Increase pro-vitamin A (PVA) content	[41]
M. acuminata	CRISPR/Cas9	*Lycopene epsilon cyclase* (*LCYε*)	Formation of α-carotene	Increase pro-vitamin A (PVA) content	[69]
M. acuminata	CRISPR/Cas9	*Carotenoid cleavage dioxygenase 4* (*CCD4*)	Causes carotenoid degradation	Increase pro-vitamin A (PVA) content	[12]
S. lycopersicum	CRISPR/Cas9	*Stay-green 1 (SGR1)*, *LCYe*, *beta-lycopene cyclase* (*BLC*), *lycopene β-cyclase 1* (*LCY-B1*), *LCY-B2*	Role in the carotenoid pathway	Increase pro-vitamin A (PVA) content	[88]
S. lycopersicum	CRISPR/Cas9	*Glutamate decarboxylase* (*GAD*)	Role in fruit development	Enhanced GABA accumulation	[4, 129]
G. max	CRISPR/Cas9	*Fatty acid desaturase 2* (*FAD2*)	Increase fatty acid content	Improved fatty acid composition	[26]
O. sativa	CRISPR/Cas9	*Vacuolar iron transporter* (*VIT*)	Regulate iron transport from between seeds and leaves	Enhanced iron content in grains	[31]
Brassica juncea	CRISPR/Cas9	*Glucosinolate transporter 1* (*GTR1*), *GTR2*	Increase glucosinolate content and defence mechanism	Attributed low seed but high leaf glucosinolate levels	[117]
B. napus	CRISPR/Cas9	*inositol 1, 3, 4, 5, 6-pentakisphosphate 2-kinase* (*ITPK*)	Synthesis of inositol-phosphate kinase 5	Reduced phytic acid content	[154]
T. aestivum	CRISPR/Cas9	*α-gliadin*	Encode almost 15–30% of storage protein in wheat seeds	Low gluten wheat	[151]
O. sativa	CRISPR/Cas-Cytosine base editing (CBE)	*Rice starch branching enzyme IIb* (*SBEIIb*)	formation of amylopectin during starch biosynthesis	Elevated-amylose content	[87]
O. sativa	CRISPR/Cas- CBE	*Nitrate transporter* (*NRT1.1B*) and *slender 1* (*SLR1*)	*NRT1.1B* is involved in uptake and transport of nitrate and *SLR1*negative regulator of gibberellin signaling pathway	Improved nitrogen utilization efficiency and dwarfism respectively	[107]
O. sativa	CRISPR/Cas- CBE	*Squamosa promoter binding protein-like* (*SPL14*)	Role in inflorescence development	Elevated grain yield	[222]
O. sativa	CRISPR/Cas- CBE	*ALS*	Involved in biosynthesis of branched-chain amino acid	Induced herbicide tolerance	[219]
Solanum tuberosum	CRISPR/Cas- CBE	*ALS*	Involved in biosynthesis of branched-chain amino acid	Induced herbicide tolerance	[180]
O. sativa	CRISPR/Cas- CBE	*Pi-d2*	Acts as an R-gene	Induced blast resistance	[148]
O. sativa	CRISPR/Cas- CBE	*BZR1* and *somatic embryogenesis receptor kinase 2* (*SERK2*)	*BZR1* regulate plant growth and development in response to brassinosteroids and *SERK2* involve in somatic embryogenesis	Elevated rice grain quality	[147]
O. sativa	CRISPR/Cas- CBE	*Wx*	Amylose synthesis	Results in amylopectin-rich rice	[101]

Table 1 (continued)

Plant	Genome editing method	Target locus	Target locus function	Trait improved	References
O. sativa	CRISPR/Cas-Adenine base editing (ABE)	*SPL14*	Role in inflorescence development	Elevated grain yield	[57]
O. sativa	CRISPR/Cas-Adenine base editing (ABE)	*SPL14* and *ALS*	*SPL14* gene play role in inflorescence development and *ALS* gene involved in biosynthesis of branched-chain amino acid	Elevated grain yield and herbicide tolerance	[56]

HKT3, *high-affinity* K^+ *transporter 7* (*HAK7*) and s*alt overly sensitive 1* (*SOS1*), endure salt stress capacity in rice [5]. Modifying the gene *related to ABI3 and VP1 2* (*RAV2*) using CRISPR/Cas technology enabled rice plants to face saline conditions [99]. Enhancements in salt stress resilience were observed in tomatoes by altering the 8-cysteine motif (8CM) and proline-rich domain (PRD) of the *hybrid proline-rich protein 1* (*HyPRP1*) coding gene [177]. Moreover, CRISPR/Cas9 targeting of genes like *drought and salt tolerance* (*DST*) in rice [153], *NAC041* [190] and *inositol 1,3,4-trisphosphate 5/6-kinase* (*ITPK1*) in barley [181] holds substantial potential for enhancing salt stress tolerance.

Improving drought tolerance

Drought stress stands as one of the most significant threats to global food security, leading to substantial losses in agricultural production and productivity. Solely, drought can cause yield reduction ranging from 50 to 70% across various crop species [77]. Following the emergence of genome editing techniques, strategies are being devised to alter genes linked to drought tolerance.

Recent breakthroughs include the identification of novel abscisic acid (ABA)-induced transcription repressors (AITRs) family, playing a crucial role in the regulation of ABA signaling and contributing to drought and salinity stress resistance in *Arabidopsis thaliana* [34]. CRISPR/Cas9-mediated targeting of the *open stomata 2* (*OST2*) gene has shown drought resistance in *Arabidopsis* [135]. Additionally, knocking out the *miR169a* gene has demonstrated notable improvement in drought tolerance in *Arabidopsis* [220]. Moreover, CRISPR/Cas9-assisted activation of the *vacuolar H+-pyrophosphate* (*AVP1*) regulating gene has been applied to enhance drought tolerance in *Arabidopsis* [137]. Further, activating the *abscisic acid-responsive element binding gene 1* (*AREB1*) [149] and silencing the *trehalase 1* (*TRE1*) gene [130] have been shown to induce drought resistance in *Arabidopsis*.

In rice plants, the modification of *enhanced response to the ABA1* (*ERA1*) gene through CRISPR/Cas9 has led to increased drought stress tolerance [132]. Likewise, CRISPR/Cas9-mediated knockouts of the *semi-rolled leaf* (*SRL*) *1*, *SRL2* and *ERA1* genes in rice have shown potential for improved drought resistance [94]. The mutation in the *pyrabactin resistance-like 9 (PYL9)* gene was proposed to enhance rice yield and drought tolerance [179]. By modifying the *DST* gene, the rice cultivar MTU1010 has been developed with broader leaves, reduced stomatal density and improved leaf water retention under drought-stress conditions [153]. In rice, genes downstream of *stress-activated protein kinase 2* (*SAPK2*), including *OsOREB1* (an ABRE binding TF), *OsRab16b*, *OsRab21*, *OsbZIP23*, *OsLEA3, OsSLAC1* and *OsSLAC7* have been modulated

using CRISPR/Cas technology for enhancement of drought stress resistance [104].

In chickpea, genes like *reveille* 7 (*RVE7*) and *4-coumarate ligase* (*4CL*), linked to drought tolerance have been edited through CRISPR/Cas9 [13]. Genome editing has been employed in maize to alter the gene to replace the *gene involved in organ size 2* (*GOS2*) promoter with an *auxin-regulated GOS8* (*ARGOS8*) promoter sequence, aiming to boost production under drought stress [161]. In tomato, CRISPR/Cas9 has targeted genes such as *gibberellin insensitive dwarf 1* (*GID1*), *lateral organ boundaries domain 40* (*LBD40*) and *mitogen activating protein kinase 3* (*MAPK3*), leading to increased drought tolerance and altered water content in tomato [103, 183]. In maize crops, the mutation in gibberellic acid biosynthetic enzyme *ZmGA20ox3* by CRISPR/cas9 results in semi-dwarf phenotype and drought tolerance [98]. Knockout of the gene *non-expressor of pathogenesis-related 1* (*NPR1*) has not only improved drought tolerance in tomato but also down-regulated drought-related genes [89].

The advancement of drought resistance in wheat has been achieved by editing *ethylene response factor 3* (*ERF3*) and *dehydration-responsive element binding protein 2* (*DREB2*) genes [70]. Likewise, CRISPR/Cas9 has been employed to modify a negative regulator of drought tolerance *3'(2'),5'-bisphosphate nucleotidase* (*Sal1*) gene in wheat, resulting in increased drought resistance in the seedling stage [1]. CRISPR/Cas-mediated targeting of the *homeobox 12* (*HB12*) gene has been reported to enhance drought resistance in cotton [48]. Furthermore, CRISPR/Cas9-mediated modification of the *repressor of GA1*-3 (*RGA*) gene in *Brassica napus* has significantly enhanced rapeseed capacity to endure drought conditions [199].

In summary, the potential of genome editing, particularly through CRISPR/Cas technology, to enhance drought tolerance in various crops is becoming increasingly evident. These efforts promise to mitigate the substantial challenges posed by drought stress to global food security. However, the ethical, regulatory and ecological aspects of genetically engineered crops need to be considered appropriately alongside these developments.

Reducing temperature sensitivity

Plants exhibit a preferred temperature range and any deviation from this range whether higher or lower can significantly hinder their growth and productivity. The response to heat stress, causing a buildup of reactive oxygen species (ROS) is regulated by heat shock proteins (HSPs) and heat shock transcription factors (HSFs). Consequently, tolerance to temperature stress in plants can be enhanced by increasing their ability to counter reactive oxygen species (ROS) [11].

The utilization of CRISPR/Cas9 technology has facilitated the creation of a cultivable rice mutant with increased heat-inducible characteristics [124]. In tomatoes, modifications using CRISPR/Cas were made to orthologs of *MAPK3* and *agamous-like 6* (*AGL6*) genes to enhance heat stress sensitivity, while *ADP-ribosylation factor 4* (*ARF4*) was used to improve sensitivity to salinity shock [22]. A positive role in heat tolerance was attributed to the *brassinazole-resistant 1* (*BZR1*) gene which promotes ROS generation in the apoplastic space of tomatoes. *BZR1* was proposed to induce the respiratory *burst oxidase homolog 1* (*RBOH1*) gene to induce hydrogen peroxide signaling for heat stress tolerance response. The mutations in *BZR1* and *RBOH1* resulted in decreased apoplastic hydrogen peroxide production and reduction in temperature tolerance showing their crucial role in heat stress tolerance [208]. In tomato mutants with reduced heat stress sensitivity are achieved through CRISPR/Cas-mediated alterations in the *heat-stress-sensitive albino 1* (*HSA1*) gene [143]. In maize, the CRISPR/Cas-mediated alteration of the *thermosensitive genic male sterile* gene was employed to generate plants that are sensitive to temperature-induced male sterility [91]. In lettuce, the knockout of *9-cis-epoxycarotenoid dioxygenase* (*NCED4*), a pivotal ABA biosynthetic enzyme allowed to germinate the seeds at a relatively higher temperature. This implies that *nced4* mutants of lettuce could hold significance in industries operating under elevated temperatures [19]. The stomatal density and photosynthesis capacity of rice have been altered by editing of the *epidermal patterning factor* (*OsEPF1*) by CRISPR/Cas9 in the rice variety ASD 16 and showed temperature sensitivity by modulating the transpiration process [145]. Calcium-dependent protein kinases (CPK) sense Ca^{2+} and are crucial for plants to exert rapid stress response against a variety of stimuli. CRISPR/Cas mediated editing of *CPK28* generated thermosensitive tomato, supporting its role in stress response [53].

To enhance the resilience against cold temperatures in plants, editing of the *MYB30* transcription factor was reported to enhance cold tolerance in rice [108]. To ascertain the precise roles of the *TIFY1a* and *TIFY1b* genes to resist cold stress, CRISPR/Cas9-mediated modification of these genes has been shown to improve yield as well as temperature resilience in rice [58]. Proline-rich proteins (PRPs) are known to have crucial roles in plants. For instance, they assist in coping with lower temperatures and also diminish the loss of nutrients, enhance the effectiveness of antioxidants, and contribute to the synthesis of chlorophyll. By utilizing CRISPR/Cas9 technology, the knockout of the *proline-rich protein 1* (*PRP1*) gene compromised rice to withstand cold conditions [125]. CRISPR/Cas9 mediated targeting of three rice-specific genes viz., *PIN5b* (an auxin efflux carrier), *grain size 3* (*GS3*) and *MYB30* showed an increase in length of the spike, larger grain and improved tolerance to cold-induced stress, respectively [215]. CRISPR/Cas9 mediated editing of genes

related to the G-complex namely, *Rice Gα* (*RGA1*), *GS3*, *dense and erect panicle 1* (*DEP1*) and *putative extra-large G protein 4* (*PXLG4*) demonstrated resistance to chilling stress in rice [36]. Due to the susceptibility to chilling stress, the fruits are prone to cold-induced damage in tomatoes. CRISPR/Cas9-based mutations in the *C-repeat binding factor 1* (*CBF1*) gene shielded tomato from cold damage by reducing electrolyte leakage [92].

Combating heavy metals stress

Oxidative stress is induced by heavy metals by stimulating the production of superoxide radicals, hydroxyl radicals (OH) and hydrogen peroxide (H_2O_2). The application of CRISPR/Cas-based genetic modification to the 5-*oxoprolinase 1* (*OXP1*) gene in *Arabidopsis* increased resilience to cadmium exposure [14]. The *natural resistance-associated macrophage protein 1* (*NRAMP1*) in rice was disabled through CRISPR/Cas9 which exhibited reduced quantities of cadmium (Cd) and lead (Pb) [35]. The CRISPR/Cas9 mediated silencing of a transcription factor *arsenite-responsive MYB1* (*ARM1*), prevented the uptake and movement of arsenic (As) in rice [184]. A novel Indica rice variety with minimal Cd accumulation in the grains has been developed by CRISPR/Cas mediated targeting of *NRAMP5* [172]. The *HAK1* (Cs^+-permeable K^+ transporter) gene governs the absorption and movement of cesium (Cs^+) in rice. By utilizing the CRISPR/Cas9 methodology, the activity of the *HAK1* was suppressed in rice [128].

Creating herbicide tolerance

The control of weed proliferation is essential to increase crop productivity. The most commonly employed method involves the use of herbicides. Herbicides not only eliminate unintended plants but also induce stress in the desired plants and weed species by disrupting or altering their metabolism. Additionally, they leave residues posing environmental risks. A primary objective in enhancing agricultural productivity is to create crop plants that possess increased tolerance to herbicides. Utilizing CRISPR/Cas9 technology to modify the *acetolactate synthase* (*ALS*) gene, has been demonstrated to develop herbicide-resistant rice, maize and watermelon [76, 166, 175, 193]. Glyphosate is a herbicide that hinders the activity of 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS) which is an enzyme involved in aromatic amino acid biosynthesis in plants. CRISPR/Cas-based targeting of the *EPSPS* gene has resulted in a glyphosate-tolerant phenotype in rice and flax [85, 155]. Recently, precise alterations through CRISPR/Cas9 in *EPSPS*, *phytoene desaturase* (*PDS*) and *ALS* genes in tomato plants have successfully induced herbicide resistance [206].

Inducing biotic stress resistance

The occurrence of plant diseases can lead to a significant reduction in both yield and the quality of crops, fruits and other edible plant products. Biotic stressors including viruses, fungi, bacteria and oomycetes are usually responsible for various plant diseases. Fungal diseases such as powdery mildew and late blight represent a severe threat to crops and significantly reduce crop yield. Many pathogens depend on distinct host genes termed susceptibility genes (S genes) for recognition, penetration and evasion of host defense. Mutations in S genes can lead to sustained, wide-ranging and heritable resistance. The *mildew resistance locus (Mlo)* gene encodes a protein that is associated with the cell membrane and possesses seven transmembrane domains. By employing the CRISPR/Cas9 technology, mutation of the *Mlo* has led to powdery mildew-resistant wheat and tomato [126, 158, 185]. Further, precise mutation in the *enhanced disease resistance 1* (*EDR1*) gene developed the powdery mildew resistance in wheat [217]. The knockout of *powdery mildew resistance 4* (*PMR4*) enhanced the powdery mildew resistance in tomato [152]. CRISPR/Cas9-based knockout of *ethylene response factor 922* (*ERF922*) [191] and *subunit of the exocyst complex 3A* (*SEC3A*) [110] genes in rice improved resistance to rice blast, but mutation in *SEC3A* gene also reported elevation of salicylic acid that caused dwarfism in rice plant. Resistance against tomato late blight (caused by *Phytophthora infestans*) was induced by CRISPR/Cas9-based multiplex targeting of *miR482b* and *miR482c* [52]. It uncovered a novel mechanism where miRNAs can be targeted by genome editing to regulate fungal resistance. CRISPR/Cas9-induced knockout of *pectate lyase* substantially reduced gray mold infection in tomato fruits [162].

Viruses present another serious threat to plants and are capable of inducing diseases in several economically important crops. CRISPR/Cas9-mediated knockout of the *eukaryotic translation initiation factor 4E* (*eIF4E*) gene created resistance against ipomoviruses and potyviruses in cucumber (*Cucumis sativus*) [28] and *Arabidopsis* [141]. The CRISPR/Cas9-mediated alteration of the *eIF4G* locus in rice showed resistance against rice tungro spherical virus (RTSV) [111]. Further, CRISPR/Cas-based targeting of *eIF4e* led to homozygous mutation and exhibited resistance against viruses belonging to the potyviridae family such as cucumber vein yellowing virus (CVYV), papaya ring spot mosaic virus-W (PRSV-W) and zucchini yellow mosaic virus (ZYMV) [141]. In a study, designing and targeting of 43 different guide RNAs specific to bean yellow dwarf virus (BeYDV) and beet severe curly top virus (BSCTV) provided resistance against these viruses in *Nicotiana benthamiana* and *Arabidopsis* [16, 62]. Tomato yellow leaf curl virus (TYLCV) specific gRNAs

from different regions like coat protein (CP), intergenic region (IR) and replication protein (Rep) were delivered in Cas9 expressing *N. benthamiana* and showed resistance to TYLCV [7]. Recently, diverse CRISPR/Cas9 tools were developed to specifically target cotton leaf curl Kokhran virus (CLCuKoV) and TYLCV [8]. The tomato mosaic virus (ToMV) resistance was induced by editing the *Dicer-like 2* (*DCL2*) gene in tomato [186]. It is reported that simultaneous editing of *DCL2a* and *DCL2b* genes enhanced resistance against the potato virus X (PVX) and ToMV [187].

Bacterial diseases also pose a remarkable threat to crop productivity. CRISPR/Cas9-based knockout of sucrose transporter *SWEET13* (S gene) enhanced bacterial blight resistance caused by *Xanthomonas oryzae* pv. *oryzae* in rice [221]. Citrus bacterial canker (CBC), caused by *Xanthomonas citri* subspecies *citri*, stands as the most prevalent bacterial threat in citrus. The alteration of *lateral organ boundary 1* (*LOB1*) promoter in Duncan grapefruit enhanced canker disease resistance [63]. The targeted CRISPR/Cas9 alteration of the *LOB1* promoter at the effector binding site (i.e., EBE_{PthA4} which enables binding of *Xanthomonas citri* subspecies *citri* efferctor PthA4), enhanced the resistance to canker disease in Wanjincheng orange (*Citrus sinensis* Osbeck) [139]. *Jasmonate-ZIM domain protein 2* (*JAZ2*) is crucial for *Pseudomonas syringae* infection in bacterial leaf spot disease. CRISPR/Cas9-based editing of *JAZ2* imparted bacterial leaf spot resistance in tomato [134]. The *downy mildew resistance 6* (*DMR6*) orthologues have been precisely modified in the banana by using CRISPR/Cas9 technology. It showed control of the pathogenicity in edited lines caused by *Xanthomonas* wilt [178]. Similarly, *DMR6* editing in tomato imparted tolerance against bacteria, oomycetes and fungi [174]. The CRISPR/Cas editing of the *DMR6* in the elite cultivar 'Italiko' belonging to *Ocimum basilicum* provides wide-spectrum resistance to downy mildew [80]. CRISPR/Cas-based mutagenesis of the *DspA/E-interacting proteins from Malus* (*DIPM*) gene within apple protoplasts provided resistance against fire blight disease [115].

Crop yield and quality are negatively impacted by pests which cause harm through both physical destruction and the spread of plant illnesses. Over the past few years, the warming climate has led to greater agricultural losses due to pests. In addition, the extensive application of pesticides can harm the environment, posing a potential drawback. The inhibition of serotonin biosynthesis through the disruption of *tryptamine 5-hydroxylase* led to an increase in salicylic acid concentrations enhancing defense against plant hoppers and stem borers in rice [106]. The utilization of CRISPR/Cas9 to create a *gmcdpk38* mutant with a *Hap3* knockout in soybean resulted in significant resistance to common cutworms [82].

Improvement of crop quality-related traits

The post-harvest loss is a major concern for a consistent supply chain throughout the year. The exploration of CRISPR/Cas9 potential is a promising way to extend the shelf life of crops. CRISPR/Cas-assisted HDR-mediated precise alteration in the *alcobaca* (*ALC*) gene has demonstrated improved tomato storage properties [79]. Similarly, employing CRISPR/Cas9 to disrupt the *pectate lyase* gene resulted in firmer tomatoes with an extended shelf life without compromising sensory and nutritional qualities [211]. Ethylene significantly influences post-harvest preservation and shelf life in climacteric fruits. Therefore, beyond targeting cell wall-degrading genes, the other effective strategy involves reducing endogenous ethylene production to delay fruit softening. CRISPR/Cas-based mutagenesis of *1-aminocyclopropane-1-carboxylate oxidase1* (*ACO1*) gene in banana delayed the ripening process by 2 days following ethephon treatment [54]. Furthermore, it was also noted to increase vitamin C and sugar content without compromising fruit quality in banana [54]. In *Solanum melongena* (eggplant), mutating the CuA-domain of the *polyphenol oxidase2* (*PPO2*) gene showed reduced browning and also increased shelf life of the genome-edited eggplant [73]. Mutation of the *squamosa promoter binding protein-like 13* (*SPL13*) gene in lettuce plants has been shown to increase biomass and leaf density [18]. The red-colored 'Ailsa Craig' cultivar of tomato was recolored into different hues including brown, yellow, pink, pink-brown, light-yellow and yellow-green by CRISPR/Cas mediated editing of three distinct genes (*PSY1*, *MYB12*, and *SGR1*) [205]

The CRISPR/Cas9 system has been employed to fine-tune the size/shape of crops in alignment with consumer preferences. Various quantitative trait loci (QTLs) and genes involved in determining the shape/size of crops have been identified. The initial QTL found to control grain length, known as *GS3*, has been effectively deactivated in five different japonica rice cultivars using CRISPR/Cas9 [160, 214] and the outcome showed elevation in grain length. Further, targeting negative regulators of grain weight (*GW2*, *GW5* and *GW6*) in rice and *GW7* in wheat enhanced the grain weight compared to the wild type [189, 201]. CRISPR/Cas mediated editing of *OVATE*, *CLAVATA* (*CLV*), *WUSCHEL* (*WUS*) and *excessive number of floral organs* (*ENO*) has been demonstrated to improves the shape and size of tomato fruits [213, 223].

The Waxy (*Wx*) gene known for encoding granule binding starch synthase (GBSS) is responsible for amylose biosynthesis. CRISPR/Cas9-based mutagenesis of the *Wx* gene has successfully demonstrated low amylose deposition while safeguarding other favorable attributes in japonica rice accessions [200]. In a parallel effort, *wx* maize mutants

have been generated across twelve high-quality inbred lines through the alteration of the *Wx* gene using CRISPR/Cas9 [44]. The diminished palatability of rice can be due to elevated grain protein content (GPC), which inversely affects eating and cooking qualities (ECQ). CRISPR/Cas-assisted editing of *amino acid permease 6* (*AAP6*) and *AAP10* within the GPC-related QTL have demonstrated the capability to swiftly reduce GPC and enhance the ECQ of rice [194]. Numerous crops possess a notable abundance of the compound 2-acetyl-1-pyrroline (2AP), imparting them aroma [183]. The betaine aldehyde dehydrogenase (BADH) plays a role in transforming γ-aminobutyraldehyde (GABald) into γ-aminobutyric acid (GABA). Therefore, *BADH2* gene mutation created through CRISPR/Cas9 has been reported to divert the conversion of GABald into 2AP and subsequently non-fragrant rice variety named ASD16 was effectively transformed into a new aromatic rice variant [10].

Improving nutrition value

Carotenoids play a role in preventing eye-related diseases and lowering the chances of cancer and cardiovascular diseases. The CRISPR/Cas9-based knock-in of *phytoene synthase* (*PSY*) genes modulated the flow of carbon into the carotenoid biosynthesis pathway resulting in enhanced β-carotene levels in rice [41]. CRISPR/Cas mediated knockout of *lycopene epsilon cyclase* (*LCYε*) and *carotenoid cleavage dioxygenase 4* (*CCD4*) genes in banana enriched the β-carotene levels in context to unedited wild type plants [12, 69]. Similarly, in tomatoes five genes related to carotenoid metabolism (*stay-green 1 or SGR1*, *LCYe*, *beta-lycopene cyclase* or *BLC*, *lycopene β-cyclase 1* or *LCY-B1* and *LCY-B2*) were targeted, resulting in five-fold enrichment of lycopene [88]. GABA serves as an inhibitory neurotransmitter, playing roles in anti-anxiety responses and blood pressure regulation. The enzyme glutamate decarboxylase (GAD) plays a pivotal role in catalyzing the conversion of glutamate to GABA. GAD features an inhibitory domain at its C-terminal, limiting GABA accumulation. To elevate GABA content, CRISPR/Cas9 assisted silencing of the *glutamate decarboxylase* (*GAD*) gene resulted in enhanced GABA accumulation in tomato and rice [4, 129]. Further, CRISPR/Cas9-mediated simultaneous targeting of *GABA-Ts* and *SSADH* resulted in an approximately 20-fold increase in GABA levels but compromised tomato fruit size and yield [83]. Monounsaturated fatty acids (MUFA) have favorable cardiovascular benefits. CRISPR/Cas-mediated mutagenesis of *fatty acid desaturase 2* (*FAD2*) has been shown to improve fatty acid composition in soybean, rapeseed and camelina [40, 65, 133]. Recently, the gene-edited soybean variety with elevated oleic acid content has been introduced for commercial availability in the United States market [26]. Enriching crop plants with micronutrients through biofortification presents a sustainable solution for individuals who are devoid of a balanced diet. CRISPR/Cas9 mediated suppression of the *vacuolar iron transporter* (*VIT*) gene elevates the iron (Fe) content in the rice grains [31]. Furthermore, the *arsenite tolerant 1* (*astol1*) rice mutant which possesses a gain-of-function characteristic, notably elevated the selenium (Se) content in the grains [165]. Recently, multiple homologs of *glucosinolate transporter 1* (*GTR1*) and *GTR2* were targeted in *Brassica juncea* (oilseed mustard) using the CRISPR/Cas system [117]. This targeted approach resulted in oilseed mustard with low-seed glucosinolate levels but high-leaf glucosinolate content while maintaining normal phenotypic attributes [154]. This study demonstrates the potential of the CRISPR/Cas system for multiplexed genome editing in oilseed mustard.

Phytic acid acts as an antinutrient by binding to minerals and proteins to form complexes. CRISPR/Cas-based editing of *inositol 1, 3, 4, 5, 6- pentakisphosphate 2-kinase* (*ITPK*) gene reduces phytic acid content in rapeseed [151]. Gluten-intolerant individuals develop coeliac disease because of gluten in wheat. CRISPR/Cas-mediated transgene-free editing of the *α-gliadin* gene imparts low gluten content in wheat grains [74].

Base editing and prime editing: modifying genome with CRISPR/Cas without DSB induction

Some of the traits bear a single-nucleotide polymorphism and can be improved by single-nucleotide alteration at the locus in the plant genome. Base editing is an innovative CRISPR/Cas tool for modifying target genes precisely either by gain-of-function or loss-of-function mutations. This can speed up the process of annotating gene functions, crop improvement and the domestication process of wild-type plants. The deaminase domain fusion version of catalytically altered Cas9 is known as the base editor that can change specific bases (A to G/C to T/C to G) directly within the genome. Hence, this process is devoid of DSBs in the genome. The base and prime editing tools along with application in plants have been described in the following sections.

Cytosine base editing

Initial cytidine base editor (CBE), CBE1 was developed through the fusion of rat cytidine deaminase (rAPOBEC1) with the N-terminus of an altered Cas9 (dCas9) containing mutations in the catalytic domain [74]. Uracil N-glycosylase (UNG) in base excision repair (BER) limits the CBE1 efficiency by removing U-G (a product of C-deamination) mismatch. UNG

inhibitor (UGI) fusion to the C-terminal of CBE1 resulted in CBE2 with better editing outcomes [74]. Replacing dCas9 with nicking Cas9 (nCas9) having one catalytic domain generated CBE3 which further improved the editing efficiency in comparison to CBE2. An additional UGI molecule fusion to CBE3 increased the UNG inhibition potential of CBE3 and was called CBE4. The protein Mu Gam from Mu bacteriophage when fused to CBE4, reduced the indel occurrence. In addition to rAPOBEC1, other cytidine deaminases such as human activation-induced cytidine deaminase (hAID), cytidine deaminase 1 (CDA1) from *Petromyzon marinus*, human APOBEC3A (hA3A), evoFERNY based editor PhieCBE and phage assisted evolved TadA-8E (an evolved version of adenine deaminase) are explored to strengthen the repertoire of CBE [150].

APOBEC1-derived CBEs were utilized for editing the *rice starch branching enzyme IIb* (*SBEIIb*) gene to disrupt an intron–exon junction and subsequently developed amylose rich rice variety [87]. Likewise, the editing of the *nitrate transporter* (*NRT1.1B*) gene led to improved nitrogen utilization efficiency, while edits to the *slender 1* (*SLR1*) gene caused significant dwarfing of rice plants [107]. Additionally, a targeted alteration in the *squamosa promoter binding protein-like* (*SPL14*) locus enhanced rice grain yield [222]. Notably, the *ALS* gene editing in wheat, rice, potato and tomato conferred resistance to herbicides in these crops [180, 219]. The *Pi-d2* (an R-gene) was effectively edited using CBE3 containing hAID and yielded blast-resistant rice [148]. The CBE3 with engineered nCas9-NG assisted alteration in *BZR1* and *somatic embryogenesis receptor kinase 2* (*SERK2*) genes showed enhanced grain quality of rice [147]. In allotetraploid cotton, the CBE3 system was used for precise point mutations in *CLA* (chloroplast biosynthetic gene) and *phosphatidylethanolamine-binding proteins* (*PEBP*) genes [142]. Further, the amylopectin-rich rice has been developed by *Wx* gene editing through *Pm*CDA1 fused with catalytically altered Cas9 from *Streptococcus canis* [101].

Adenine base editing

The adenine base editor (ABE) is nCas9 (D10A) and adenosine deaminase enzyme fusion which facilitates the conversion of adenine (A) to inosine (I) within the target DNA sequence. I:T base pair is finally converted to G: C base pairs in subsequent repair and replication events. First ABE (ABE7.10) was composed of TadA and TadA7.10 (engineered adenine deaminase) dimer fused to nCas9. A nuclear localizing signal (NLS) was added to ABE7.10 to improve its efficiency. Another NLS when added to both ends, ABEmax was generated which has been shown to efficiently edit *Acetyl-CoA carboxylase* (*ACC*) [93], *MAPK6*, *SERK2* and *WRKY45* genes in rice [203]. Targeted modification of A·T to G·C base pair in the *SPL14* gene increased grain yield in rice [57]. Targeted ABE-assisted editing of *SPL14* and *ALS* genes in rice was reported to enhance yield and herbicide tolerance, respectively [56]. Subsequent advancement included the more efficient editing of *Wx* and *ALS* genes in rice, achieving editing frequencies up to 100% through the utilization of TadA8e-based ABE8e [195]. The engineered version of TadA8e (having a single-stranded DNA-binding domain) in PhieABE showed efficient editing in the broader window in comparison to other ABEs [169]. A more potent TadA9 was engineered from TadA8e and has been demonstrated to efficiently edit challenging endogenous targets [204]. These successful advancements and applications of ABEs in plants showcase immense potential in advancing biological research and the engineering of crop plants, leading to the development of improved traits.

The C to G base editing

The C to G base editor (CGBE) introduces a novel dimension to the existing landscape of base editing. The CBE and ABE primarily perform transitions of bases rather than transversions. However, CGBE is considered a recent innovation that has overcome the limitations of CBE and ABE.

The CGBE is comprised of nCas9 (D10A), rAPOBEC1 cytidine deaminase and UNG which can efficiently introduce base transversions [78]. A CGBE composed of the codon-optimized UNG (OsCGBE03) facilitates C-G editing at five different endogenous loci in rice including *ideal plant architecture 1* (*IPA1*), *bZIP5*, *SLR1*, *ALS1* and *NRT1*.1 [176]. Further, engineering of the TadA-8e enzyme (N46L) diminished its adenine deaminase activity and has the potential to generate precise C to G base editing. Overall, CGBE expands the base editor repertoire and is considered a potent tool in the context of precise crop breeding and improvement.

Prime editing: search and replace

A revolutionary search and replace genome editing strategy called "prime editing" enables precise modifications in the genome without needing DSB. The fact that prime editors can incorporate point mutation, transversion, transition, deletion and insertion mutations of up to 50–80 bp without additional donor DNA templates. A modified nCas9 (D10A) C-terminal fusion with reverse transcriptase (RT) forms prime editor (PE). Moloney murine leukemia virus reverse transcriptase (M-MLV-RT) has been specifically used in the designing of PE. In prime editing, a guide RNA termed prime editing guide RNA (pegRNA) is involved which consists of a traditional single-guide RNA (sgRNA) known as reverse transcript encoding the desired edit (RTT) and a primer binding site (PBS) that initiates the reverse transcription process. The PE interacts with the target DNA and introduces a nick on the non-target strand. The 3' terminal of DNA aligns with the PBS, initiating reverse transcription and incorporating the intended

edit into the genomic DNA. Subsequently the edited DNA is replicated and repaired. The first generation of PE (PE1) was the fusion of nCas9 (H840A) and M-MLV-RT. Further, the replacement of M-MLV-RT with its engineered version led to the formation of PE2. The use of addition nicking gRNAs further improved the efficiency in PE3 and PE3b. In PE4 and PE5 for suppression of DNA mismatch repair, the dominant negative mismatch repair protein was fused to PE that significantly improved its activity. Engineering of nCas9 in PEmax surprisingly improved editing outcomes. For deletion and insertion of large fragments, TwinPE and GRAND editor were composed of two specifically designed pegRNA [9, 188].

The Plant prime editors (PPE) viz., PPE2 and PPE3 (or PPE3b) have shown efficient editing at *CDC48*-locus in wheat and *CDC46* as well as *ALS* in rice. This breakthrough has significant implications for developing herbicide-tolerant crops and advancing functional genomics studies [96]. The rice genes *ACC2* to *ACC4*, *PDS2* to *PDS6* and *Wx* are also targeted by using the prime editors. *ACC2* to *ACC4* and *PDS2* to PDS6 genes have shown promising mutations but in the *Wx* gene, mutation was not detected [96]. These studies have shown variation in mutation efficiency at various targeted loci, thus a need to validate pegRNA by using *in-vitro*/in-*vivo* assays. The improved editing efficiency in maize has been shown by enhancing the expression of pegRNA targeting for the *ALS* gene [64]. In this case, a PE system with two pegRNA was developed for editing *ALS1* and *ALS2* in maize and demonstrated herbicide resistance carrying the P165S or the W542L/S621I mutation in *ALS1* and *ALS2*, respectively. This showcases the adaptability of prime editing in plants for studying gene functions and enhancing crop resilience and yield.

In addition to monocots, prime editors have also been employed in dicot plant species (tomatoes and potatoes) [51]. The potential of prime editors can also be explored to edit other genes for improving the nutritional (high amylose, β-carotene and oleic acid contents) and agronomic (yield, texture, etc.) traits simultaneously.

Challenges associated with genome editing

Genome editing holds significant promise for advancing plant trait development. However, it necessitates improvement to overcome several inherent limitations. Some of the major bottlenecks in genome editing are discussed here.

Factors hampering CRISPR/Cas-based genome editing

In the realm of plant genetic engineering, the common practice involves introducing foreign genes into plants through *Agrobacterium*-mediated transformation, where these genes become stably integrated into the plant genome [45]. However, the removal of transgenes following genome editing is typically limited to sexually propagated crops. The integration and sustained expression of genome editors may contribute to the occurrence of off-target mutations [122]. Additionally, the presence of foreign DNA raises concerns related to biosafety and regulatory considerations.

It's worth noting that many elite crop varieties and inbred lines, which are commonly used in commercial breeding, exhibit resistance to conventional transformation methods. This poses a challenge for techniques such as biolistic bombardment and regeneration from protoplasts, which are commonly employed in the genome editing process [146] The transformation protocols independent of different genotypes are needed to explore the potential of genome editors in plants. As a result, finding effective means of introducing genetic modifications in these elite crops remains a notable obstacle in the field of genome editing-mediated engineering of plants.

Low efficiency of HDR-based gene knock-in

CRISPR/Cas-based gene replacement practices are limited to plant systems due to naturally lower rates of homologous recombination. NHEJ resulting in gene knockouts is the predominant repair mechanism in plants over the HDR pathway, the presence of donor template in the S and G2 phase of the cell cycle is crucial for successful gene knock-in [61, 140]. Also, inefficient delivery methods for knock-in reagents limit the gene knock-in using the HDR pathway. Indeed, the optimization of donor DNA and optimal expression of gRNA and Cas9 are also essential requirements for effective gene replacement by using HDR-mediated gene knock-in [167].

Challenges associated with base and prime editors

Besides the great potential, base editors led to the generation of off-target mutations. Moreover, base editors can only induce transition mutations and cannot facilitate base transversions. Their limitation of strict editing window is also a major concern for their restricted applicability [84]. Prime editing, a noteworthy development in plant genome editing, addresses this limitation by enabling base transversions/insertions/deletions at target loci. However, prime editing exhibits relatively low efficiency, particularly in dicot species with lower editing frequencies observed for insertions compared to deletions and substitutions [95, 202].

Recent advancements and opportunities for addressing bottlenecks in plant genome editing

CRISPR/Cas-based genome editing has tremendous potential to modify plant genomes with precision. Recent advancements help in addressing longstanding bottlenecks

limiting the widespread applications of the CRISPR/Cas system.

Designing and screening of gRNA

The effectiveness of editing constructs can vary significantly, specially when multiple genes are simultaneously targeted using more than one guide RNA (gRNA). Further, the editing efficiency of each gRNA while multiplexing is not uniform [60]. Various systems have been devised to assess the validity of constructs before their stable editing in plant tissues. These systems include *in-vitro* and *in-vivo* cleavage of the target DNA sequence in various plant tissues (protoplasts, cell suspension, hairy roots, leaf epidermis) with ribonucleoprotein (RNP) complex using biolistic, electroporation and polyethylene glycol (PEG) mediated delivery methods [24, 72, 127]. Testing gRNA efficiency before employing it in stable genome editing of crop plants not only improves editing efficiency but also saves time and resources.

Emerging Cas variants for advanced genome editing

Cas9 nuclease stands as the most widely used Cas effector in genome editing. However, recent studies have shown the emergence of new Cas variants such as Cas12a/Cpf1 for DNA targeting and Cas13 for RNA targeting. These Cas variants displayed advantageous features and overcame several limitations of traditional CRISPR/Cas9 system. Some of the important Cas variants belonging to different classes are described here along with their promising applications in plant genome editing.

Cas12a/Cpf1

Cas12a/Cpf1 is derived from *Prevotella* and *Francisella* bacteria. It is smaller than Cas9 and possesses nuclease (NUC) lobe with two RuvC-like domains. Upon activation through base pairing, it cleaves both target/non-target DNA strands, producing staggered ends in a T-rich PAM-dependent manner. Cas12a, the pioneering Cas12 nuclease for genome editing, processes pre-crRNA into mature crRNA independently of tracrRNA [216]. The CRISPR/Cas12a system facilitates gene insertion, deletion, tagging, and base editing in economically vital plants. Comparative studies in rice targeting the *epidermal patterning factor like-9* (*EPFL9*) gene indicated that the CRISPR/LbCpf1 (LbCas12a) system outperforms Cas9 by increasing mutation percentages and larger deletions [207]. The single transcript unit (STU)-Cas12a system has been designed for single/multiplexed rice genome editing [173]. Additionally, the catalytically dead Cpf1 serves as a transcriptional repressor in plants and bacteria, indicating its potential to regulate plant transcriptomes [171]. Recently, Cas12a orthologs (Hs1Cas12a and Ev1Cas12a) have shown their tremendous potential in both monocot and dicot plant genome editing [90]. The efficient editing of multiple loci and the generation of heritable mutations using the CRISPR/Cas12a system hold promise for developing crops with enhanced yields, disease and pest resistance and other desirable traits.

Cas12b

Cas12b possesses a conserved RuvC-like and a putative NUC domain that are significantly different from those found in Cas12a. CRISPR/Cas12b/C2c1 utilizes a hybridized gRNA formed through the combination of crRNA and tracrRNA to guide its endonuclease activity [97]. Moreover, Cas12b is a more compact, efficient and convenient tool dependent on VTTV (where V is A/G/C) PAM, contributing to editing efficiency exceeding 50%. Notably, Cas12b stands out as the sole Cas protein that generates the longest nucleotide overhangs (6 to 8 nucleotides) in the staggered end, instead of the 1 to 3 nucleotides observed in Cas9 [182]. This characteristic proves advantageous as it decreases errors while NHEJ-mediated repairing. The versatility of Cas12b in functioning across a wide range of temperatures and pH levels facilitates more effective functional studies, especially for robust crops with heat- and salinity-tolerant traits. Cas12b proteins from various bacteria, particularly AaCas12b (*Alicyclobacillus acidiphilus*), demonstrated improved mutation specificity in rice [120]. AaCas12b has shown effective functionality at high temperatures, making it a promising candidate for developing heat-tolerant crops, as observed in cotton [182]. Other Cas12b types, like BhCas12b v4 (*Bacillus hisashii*) and BvCas12b (*Bacillus* sp. V3-13) in *Arabidopsis*, exhibited high potential for multiplex genome editing and heritable mutations [198].

Cas13

Cas13 represents a category of RNA-guided ribonucleases with a specific focus on targeting RNA. Unlike Cas9 or Cas12, which rely on a PAM for target recognition, certain Cas13 proteins exhibit a preference for a protospacer flanking site (PFS) [131]. All identified Cas13 nucleases feature two distinct higher eukaryote and prokaryote nucleotide-binding (HEPN) domains in the NUC lobe for precise RNA cleavage [131]. Cas13 holds significant potential in diverse applications within plant research, including targeted RNA knockdown, defense against RNA viruses, and modification of the epitranscriptome. LwaCas13a in rice protoplasts resulted in more than 50% knockdown for seven out of nine tested gRNAs [2]. LshCas13a has shown promise in

conferring immunity against RNA viruses in both monocot and dicot plants, offering a potential avenue for developing disease-resistant crops using CRISPR technology [159]. Notably, Cas13d, a subtype targeting RNA molecules without a strict PFS preference, recognizes the uracil base within the target RNA, allowing it to target a broader range of RNA molecules. Cas13d is found to effectively function across a wide temperature range, making it suitable for highly sensitive nucleic acid detection methods like reverse transcription recombinase polymerase amplification (RT-RPA). Additionally, the inactivated form, dCas13d retains its target-RNA-binding capacity and when fused with a modified plant APEX2, it enables the detection of RNA–protein interactions [21]. These properties collectively position Cas13d as a potent tool in transcriptome engineering.

Class 1 type 1 CRISPR/Cas system

Cas3, the distinctive protein associated with the type I CRISPR system, functions as a helicase-nuclease with a histidine-aspartate (HD) nuclease domain [196]. Although Cascade-Cas3 has been extensively utilized for prokaryotic genome modification, its application in eukaryotes faced challenges due to the requirement for multiple-subunit effectors, necessitating the simultaneous/sequential expression of multiple genes. However, the advancements led repurposing of Cascade-Cas3, overcoming this limitation. In the well-studied type I-E CRISPR/Cas system, the Cascade complex includes five Cas proteins (Cas5e, Cas6e, Cas7e, Cas8e and Cas11e) and crRNA. Upon PAM interrogation by Cas8, crRNA and the target DNA form an R-loop, followed by the recruitment of the specific nuclease Cas3, resulting in cleavage and degradation of the target DNA [50]. While the type I-E system has been applied for transcriptional control in maize [209]. Recently explored type I-D CRISPR/Cas system, TiD, from *Microcystis aeruginosa* has been optimized for eukaryotic genomic editing. TiD exhibits a unique combination of type I and type III effector modules, featuring a hybrid helicase (Cas3') and an HD nuclease domain (Cas3") fused with Cas10d. This hybrid nature qualifies TiD as a robust genome editing tool for complex crop genomes, offering potential benefits in crops like cassava, wheat, *Brassica* and potato for improving traits such as enhanced nutritional value and disease resistance [68, 109]. The distinct features of class I CRISPR/Cas prevalent in complex prokaryotes, make them promising and advantageous for enhancing plant traits through DNA editing.

CasΦ

CasΦ system represents a highly compact tool for genome editing. It is characterized by a reduced number of spacers in its CRISPR array, lacking the CRISPR spacer acquisition machinery (Cas1, Cas2 and Cas4) [138]. The CasΦ protein, with its small size (70–80 kDa) is conveniently packaged in a viral vector, facilitating the straightforward and effective transgene expression [138]. In the realm of plant genome editing, CasΦ-2 demonstrated the ability to induce 8–10 bp deletions in the *phytoene desaturase 3* (*PDS3*) gene in *Arabidopsis*, indicating robust editing capability [138]. Subsequent enhancements of the CRISPR-CasΦ-2 system in tobacco and *Arabidopsis* have resulted in better specificity and efficiency of genome editing [25]. This optimized system holds promise for application in economically significant crops to enhance their desirable traits.

Addressing the challenges in base and prime editing

Efficient strategies for improving base editing include mitigating gRNA-independent off-target effects by the utilization of alternative deaminases or implementing modifications to the deaminase protein [210]. Improved CBE variants, like YEE-BE3 have been proposed as potential solutions to reduce off-target edits in plants [66]. Despite advancements, base editing faces challenges such as restricted target selection due to PAM site compatibility and editing window length limitations [84]. To overcome these constraints, different Cas orthologs and modified variants with changed PAM specificities have been utilized [147, 192].

Researchers have developed strategies including engineered prime-editing proteins, manipulation of the mismatch repair pathway, improved guide RNA design, and optimization of delivery methods to enhance prime editing efficiency [95]. The careful design of the pegRNA, emphasizing the selection of a suitable combination of the PBS and RT template is crucial for achieving high efficiency [55]. Despite these efforts, challenges persist in target gene selection and navigating plant transformation steps, particularly in the context of large plant genomes with duplicated regions and genes. In addition, the optimizations of genome editing reagents, gene delivery systems and transformation protocols are essential for achieving efficient prime editing in plants.

Improving delivery method for CRISPR/Cas-based genome editing reagents

The efficient delivery of GE reagents may enhance the editing efficiency in desired plant systems. Till now, two types of delivery methods for CRISPR components have been reported which are mainly physical (microinjection, electroporation, PEG, mechanical cell deformation) and biological (Agrobacterium-mediated, viral vector-based transformation systems [164]. *Agrobacterium*-based binary

vectors are widely used for plant cell transformation to achieve knock-out and knock-in mutations. In *Arabidopsis,* HDR-based CRISPR/Cas editing was achieved at *repressor of silencing 1* (*ROS1*) and *Demeter* (*DME1*) loci via sequential *Agrobacterium*-mediated transformation in Cas9 overexpressing plants [119]. In viral vector-mediated transformation, geminivirus replicons (GVR) are used as gene editing vector. GVR is a single-stranded DNA-based vector having a genome size of 2.5 kb including replication proteins (Rep/RepA), histone protein, long intergenic region (LIR), short intergenic region (SIR) and the origin of replication (ori) that is triggered by a single bidirectional promoter [37]. The CRISPR/Cas gene editing by the bean yellow dwarf viral vector (BeYDV) delivery system was achieved in tobacco by targeting the *ALS* gene [15]. In tomato, BeYDV vector used for targeted insertion of cauliflower mosaic virus 35S strong promoter (CaMV35S) at the upstream of the *anthocyanin I* (*ANTI*) mutant gene has shown purple coloration in tomato [27]. The tobacco rattle virus (TRV) is used to deliver gRNAs into tomato cultivar Micro-Tom expressing Cas9 or co-delivery of Cas9 and sgRNAs by potato virus X (PVX) vector targeting *PDS* gene. The cotton leaf crumple viral (CLCrV) vector is used to edit *GL2*, *BRI1*, *PDS* genes and GUS transgene in *Arabidopsis* by designing gRNA fusion with mobile sequences like Flowering Locus T (FT) mRNA at the 5′ end [81]. Furthermore, the multiplex editing using the TRV vector in *Arabidopsis* has been optimized by targeting *magnesium-chelatase subunit 1* and *subunit 2* (*AtCHLI1*, *AtCHLI2*) genes simultaneously [123]. Another tripartite RNA virus, barley stripe mosaic virus (BSMV) has been engineered to deliver editing reagents by agroinfiltration methods in maize and wheat crop plants. In wheat crops, the editing of the *histidine-rich calcium-binding* (*HRC*) gene for *Fusarium* head blight (FHB) resistance, improved FHB resistance in wheat [32]. In barley crops, virus-induced genome editing (VIGE) mediated by BSMV vector in transgenic barley overexpressing Cas9 plants is also shown. The CRISPR/Cas editing at target locus *albostrians* gene (*CMF7*) by BSMV vector resulted in transgenic barley having variegated/albino chloroplast phenotypic mutation. Furthermore, *MUS81* (a DNA structure selective endonuclease), *ASY1* (an axis-localized HORMA domain protein) and *ZYP1* (a transverse filament protein of the synaptonemal complex) are also edited by CRISPR/Cas mediated editing by BSMV vector [168].

The TRV RNA viral vector is also used for base-editing where gRNAs targeting to *cloroplastos alterados 1* (*CLA1*) and *PDS3* genes were targeted using cytidine deaminase base-editor in *Arabidopsis* plants [100]. These examples have shown the immense potential of viral vector-mediated efficient genome editing in plants.

Potential use of morphogenetic regulators in plant genome editing

Some developmental regulators (DRs) such as WUSCHEL (WUS) and BABYBOOM (BBM) are demonstrated to induce somatic embryos when ectopically expressed and expand the scope of genome editing in recalcitrant plant species. The co-expression of *Wuschel 2* (*Wus2*) and *isopentenyl transferase* (*ipt*) morphogenetic genes along with genome editing components in soil-grown plants led to induced *de-novo* meristem formation that subsequently rose to genome-edited shoot in tobacco, grape and potato plants [112]. Hence, the escaping of the tissue culture practice in this way can simplify the genome editing process. The co-delivery of morphogenetic regulators with genome editing reagents significantly improves the regeneration of transgenic plants [47]. In the B104 public maize inbred variety, the co-expression of *WUS* and *BBM* genes by using stage-specific promoters (phospholipid transfer protein and auxin-inducible promoters) resulted in enhanced somatic embryo formation. For the controlled expression of DRs, Cre/LoxP recombination system along with the selection marker *ALS* gene resulted in enhanced transformation efficiency. Moreover, editing by CRISPR/Cas system was also validated with DRs system by targeted mutation in *virescent yellow-like* (*VYL*) gene [49]. The seedlings with an increased number of leaf trichomes were produced by editing of the *teosinate branched 1/cycloidea/proliferationg* cell *factor 4b* (*TCP4b*) gene in a *WUSa* overexpressing transgenic *Brassica rapa* plant [102].

Further, due to the pleiotropic nature of developmental regulators, their prolonged-expression may cause abnormalities in plants. However, controlling the expression of these regulators by using a chemical inducible system and stage-specific promoter offers a more sustainable and effective approach to achieving effective regeneration of genome-edited progeny [105]. In poplar genome editing, the activation of endogenous morphogenetic genes, like *WOX* 11 and *WUS* via CRISPR/Cas showed enhanced regeneration efficiency [136]. Recently, recombination-based transgene removal followed by selection marker activation has demonstrated a sustainable method for boosting regeneration in sorghum genome editing [30]. In the Sorghum crop, the combinatorial effect of growth-stimulating factor 4 (GRF4) fusion with GRF-interacting factor1 (GIF1) along with helper plasmid pVS1-VIR2 resulted in maximum transformation efficiency up to 38.28%. [86]. Furthermore, the repertoire is expanded by recently discovered morphogenetic regulators, WOX6 and GRF4-GRF-fusion [29, 38]. Moreover, GRF5 expression improves maize and sugar beet regeneration efficiency [75]. Therefore, the expression of morphogenetic genes increases the efficiency of regeneration and offers a

straightforward method to increase the current capacity of genome editing in recalcitrant plant species.

CRISPR/Cas assisted chromosomal engineering

Plant breeding relies on genetic variation and the ability to manipulate genetic linkages between traits. However, these linkages pose a significant challenge to transferring desirable traits from wild species to cultivated relatives. The development of CRISPR/Cas technology has empowered breeders to introduce genetic variability in a controlled and site-specific manner, enhancing traits with high efficiency. Recent studies in *Arabidopsis* and maize have reported the successful induction of large-scale chromosomal rearrangements [20, 157]. The targeted DSB induction using Cas9 has induced recombination between homologous chromosomes in somatic cells of tomatoes, resulting in gene conversions and putative crossovers [17]. Moreover, the targeted inversion of up to 18 kb was successfully induced in *Arabidopsis* using Cas9 from *Staphylococcus aureus* under egg-cell-specific expression [156]. Further evolutionary-derived inversion in *Arabidopsis* and an elite maize inbred line were reversed using the CRISPR/Cas system. This CRISPR/Cas-mediated inversion in a crop plant, specifically in an elite maize inbred line, spanned nearly one-third of chromosome 2 [157]. Also, the large translocations commonly found in crops can reduce meiotic recombination. The first targeted induction of reciprocal translocations in plants was in *Arabidopsis* between chromosomes 1 and 2, and between chromosomes 1 and 5 [20]. These translocations were heritable with fragments around 1 Mb and 0.5 Mb in size. Despite these challenges, the CRISPR/Cas system holds tremendous potential in chromosomal engineering, paving the way for the production of designer crops with desired chromosomal structures shortly.

Regulatory landscape of genome editing crops: current status and future

The environmental release of genome-edited crop plants will be determined by the adoption of appropriate biosafety regulatory guidelines or policies in various countries. The regulatory framework for genome-edited crops is rapidly changing worldwide by considering science-based policies and the legality of releasing the crops onto the market. The current status of the global regulatory landscape for genome editing crops can easily be accessed from a publicly available resource "Global gene editing regulation tracker" (https://crispr-gene-editing-regs-tracker.geneticliteracyproject.org/). In India, genome editing-derived plant products free from exogenous foreign DNA and fall under SDN-1/SDN-2 categories are exempted from strict biosafety assessment (https://pib.gov.in/PressReleasePage.aspx?PRID=1871153). However, SDN-3 involves the precise insertion of a donor DNA repair template or foreign gene into the genome of crop plants is considered under the genetically modified organisms (GMOs) category and regulations [140]. The United States of America (USA), Canada, and South American countries also classified genome-edited crops into three categories and considered SDN-1 and SDN-2 as conventional breed crops. The USA has secured no unique regulation status and biosafety assessment for these two categories. Recently, in 2023 Environmental Protection Authority (EPA) in the USA added safety requirements to the current SECURE Biotechnology regulations of the United States Department of Agriculture (USDA). In a similar direction, several other countries including Israel, Argentina, Brazil, Chile, Colombia, Paraguay, Ecuador, Japan and Australia have chosen to exempt genome-edited plants from GMO laws by the case-to-case study as long as no foreign DNA is incorporated into the plant genome [23].

Contrarily in the case of the European Union (EU), the European Court of Justice (ECJ) classified genome-edited lines as GMOs, prohibited for cultivation and consumption. However, the EU is reassessing its genome editing regulation status and has proposed new regulatory guidelines where the genome-edited plants with no foreign DNA (new genomic technology 1: NGT1) will not be regulated, while plants with foreign DNA (NGT2) will be treated as transgenic [23]. New Zealand is regulating genome editing by considering them under GM biosafety rules. Although the EPA of New Zealand took interest in the formulation of genome editing regulations there is still no clear path for the cultivation and commercial release of relevant products [23]. The United Kingdom has exempted genome-edited crops from the GMO definition, and possibly allowed field trials to the commercial release of these crop plants [42]. The Ministry of Agriculture of China published guidelines in January 2022 for the safety assessment of genome-edited plants that are free from exogenous DNA [114]. In summary, most of the countries have considered less strict regulation of genome-edited crops with the requirements of key information such as targeting trait/gene, stability of trait, possible associated risks and benefits, method of generation, and evidence of lacking vector backbone/foreign DNA. Moreover, the legislation and regulations about gene-edited crops are rapidly evolving and adapting to new technologies. These developments are crucial in facilitating the entry of gene-edited products into the market (Table 2) and raising public awareness about the benefits of this technology.

Table 2 Commercialization status of genome-edited crops worldwide. *Source* Biotech Updates from ISAAA.org; https://www.isaaa.org/kc/cropbiotechupdate/newsletter/default.asp)

Genome edited crop	Improved trait	Company/Country	Status (Commercialized/ Noncommercialized)
Soybean	Insect resistance	Shandong Shunfeng Biotechnology Co, China	Commercialized, 2023
Maize	Herbicide tolerance	Shandong Shunfeng Biotechnology Co, China	In the field trial
Potato	Reduces browning and increases shelf life	Balcarce Agricultural Experimental Station, Latin America	In field trials
Tomato	High antioxidant	Norfolk plant sciences, United States of America	Commercialized, 2023
Teff, a native grain of Ethiopia	Semi-dwarf teff	Donald Danforth plant sciences, United States of America	In field trials
Banana	Reduced browning and increased shelf life	Tropic, United Kingdom	In regulatory process
GABA enriched tomato	Increased gamma-aminobutyric acid (GABA) in tomato	Sanatech Seed, Japan	Commercialized, 2022
Maize	High starch component	Japan	In regulatory process
Wheat	Reduced amino acid (asparagine) content	United Kingdom	In field trials
Mushroom	Reduced browning	United States of America	Commercialized

Conclusion and future perspective

The capability to edit multiple genes makes CRISPR/Cas an attractive option for enhancing various traits simultaneously. CRISPR/Cas9 mediated new breeding tool provides substantial benefits compared to traditional plant breeding methods. It allows a short duration for the introduction of desirable traits in a precise manner, while conventional approaches usually take a long time (around 6 to 7 years) and also carry undesirable traits/effects [59]. Therefore, to meet the quality food requirements of an exponentially growing population in changing climatic conditions, CRISPR/Cas9-based editing holds high promise. Plant tissue culture is one of the crucial limiting factors to genome-editing experiments. Further, the long exposure to culture under *in-vitro* conditions may also induce somaclonal variations, which hampers the widespread application of crop improvement practices. Recent reports showed that the implication of morphogenetic regulators allows direct regeneration of edited shoots [112]. The implication of plant morphogenetic regulators such as *BBM*, *WUS* and *IPT* have been used for improving plant transformation efficiency [29, 112]. Newly emerging tools such as CRISPR-Combo can activate and suppress gene expression simultaneously and show potential application in plant metabolic engineering [136]. The incidence of unintended off-targets also limits the potential of the CRISPR/Cas system. Engineering of Cas9 endonuclease as well as exploration of robust and highly specific Cas orthologs, such as FnCas9 may be the solution to this stumbling block [3]. Moreover, genome editing outcome mostly relies on NHEJ for repairing DSBs instead of HDR repair which in turn results in random insertion or deletions. Using strategies like inhibiting NHEJ and overexpressing HDR components may improve HDR-based precision genome editing [163]. The comprehensive knowledge of genomic sequences and annotation helps to predict the editing outcomes at the loci of interest. As of now, only certain crop plant genomes have been fully sequenced. The innovations in omics technologies are certainly adding information to databases that will be crucial for crop improvement programs. The evolving regulations worldwide are attracting researchers towards genome editing application for crop improvement programs.

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Declarations

Conflict of interest The authors declare that they have no conflicts of interest.

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