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Review Article

Cereal crop genome editing tools and their applications to sustainable agriculture

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Abstract

Sustainable agriculture is essential to attain food security and address increasing climate change concerns. Population, abiotic and biotic stresses and limited agricultural water supplies and land are important barriers to cereal crops production, in addition to extreme weather. These factors influence their quality and productivity. Therefore, there is a pressing need for effective methods of food production under these conditions. For many individuals in low- and middle-income nations, cereals represent a key food source. Developing cereal crops with enhanced flexibility, high yields, and the ability to tolerate these biotic and abiotic problems is therefore essential. Modern OMICS techniques, next-generation sequencing, bioinformatics tools and the most recent improvements in genome editing tools (GET) have made targeted mutagenesis conceivable. By altering a crop variety's endogenous genome, which is free of any foreign genes, genome editing (GE) enhances the crop variety. Therefore, crops that have undergone GE but have not integrated foreign genes are not considered genetically modified organisms (GMOs) in a number of countries. GE is being used by researchers to promote the nutritional value of cereal crops and increase their tolerance to biotic and abiotic stresses. This review critically discusses the activities of GET, the role of bioinformatics tools and its application to sustainable agriculture for cereal crops.

Keywords: bioinformatics tools; cereals; CRISPR; genome editing; gRNA design

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Introduction

Agriculture's most pressing issues are increasing food production while using the same amount of resources (land and water), improving nutrition, and supporting farmers with climate change adaptation (Searchinger *et al*., 2019). Cereal crops are the most significant food and nutritional sources, as they supply fuel, feed, and other consumable resources for human life (Awika, 2011). Most countries get more than 30% of their calories from cereal crops, including rice, wheat and maize (Shiferaw *et al*., 2011; Shallan *et al*., 2010a,b). Cereals are edible grains and are members of the Poaceae (Gramineae) family of plants (Barak, 2018; Borlaug, 1998). The Poaceae family includes three main subfamilies: Panicoideae (sorghum and maize), Oryzoideae (rice), and Pooideae (rye, wheat, oats and barley) (Maughan *et al*., 2019). Sustainable cultivation of cereal crops is necessary for global food security (Singh *et al*., 2016).

Mutation breeding, cross breeding and gene transfer are currently the main plant improvement techniques used in modern agriculture. It takes several years to generate genetic diversity and introduce beneficial alleles through cross-breeding (Scheben *et al*., 2017). Because of this, the traditional methods of plant improvement, such as cross-breeding or random mutagenesis, are time-consuming and unable to keep up with the rising global need for food. In addition, in the process of gene transfer, if a gene sequence is randomly inserted into the genome of a plant, it may disrupt the gene at the site of insertion and the new gene product may also interfere with the function of other molecules already present in the cell (Vogan and Higgs, 2011). Moreover, lengthy and expensive governmental examination procedures as well as public concerns restrict the commercialization of genetically modified plants (Prado *et al*., 2014).

Recently, modern genome editing tools (GET) have the prospect of precisely integrating a foreign gene into a specific site of the plant genome, enabling the specific nucleotide replacement of the target sequences (Feng *et al*., 2013; Abdelrahman *et al*., 2018). Particularly for crops with complicated genomes that are challenging to enhance through traditional breeding techniques, GE has emerged as an incredible option for effective and focused genome changes (Feng *et al*., 2013). The inherent properties of each nuclease and target genome affect the competence, precision, and mutation structure of the outputs of genome engineering. Inducing gene insertions, gene replacements, or insertions or deletions that interfere with a particular gene's function is possible using GET (Hsu *et al*., 2014). With the aid of GE tools, it is possible to make precise changes at specific genomic locations, replace or modify an existing allele derived from the wild or insert new genes (Zhu *et al*., 2017; Kamburova *et al*., 2017). The aim of this review was to discuss the different tools used in cereal genome editing (GE) and their different applications.

Genome editing tools

GET including meganucleases (MNs), zinc-finger nucleases (ZFNs), transcription activator-like effector nucleases (TALENs), and clustered regularly interspaced short palindromic repeats/CRISPR-associated protein (CRISPR/Cas9). GER enable specific genetic alterations by inducing specific DNA double-strand breaks (DSBs) (Figure 1). MNs are naturally occurring small DNA cleavage enzymes and can recognize long (12-40 base pairs) DNA targets. They are encoded by introns or mobile genomic elements. Smith et al. (2006) used meganucleases with target sites up to 18 bp in length to produce the first genome editing success; however, the position of the DSB is defined by the enzyme's inherent selectivity, which is difficult to change. The solution to this problem was to create nucleases with specific specificity. ZFNs contain the FokI restriction endonuclease cleavage domain and a Cys2-His2 zinc-finger protein. TALENs contain the DNA-binding TALE amino-terminal domain and FokI cleavage domain. The two primary examples are ZFNs, which pair numerous zinc-finger DNA-binding domains with a promiscuous endonuclease domain that recognises a 3-bp

module (Kim *et al*., 1996) and TALENs, which link numerous transcription activator-like effector domains that recognize single base pairs with a promiscuous endonuclease domain (Christian *et al*., 2010).

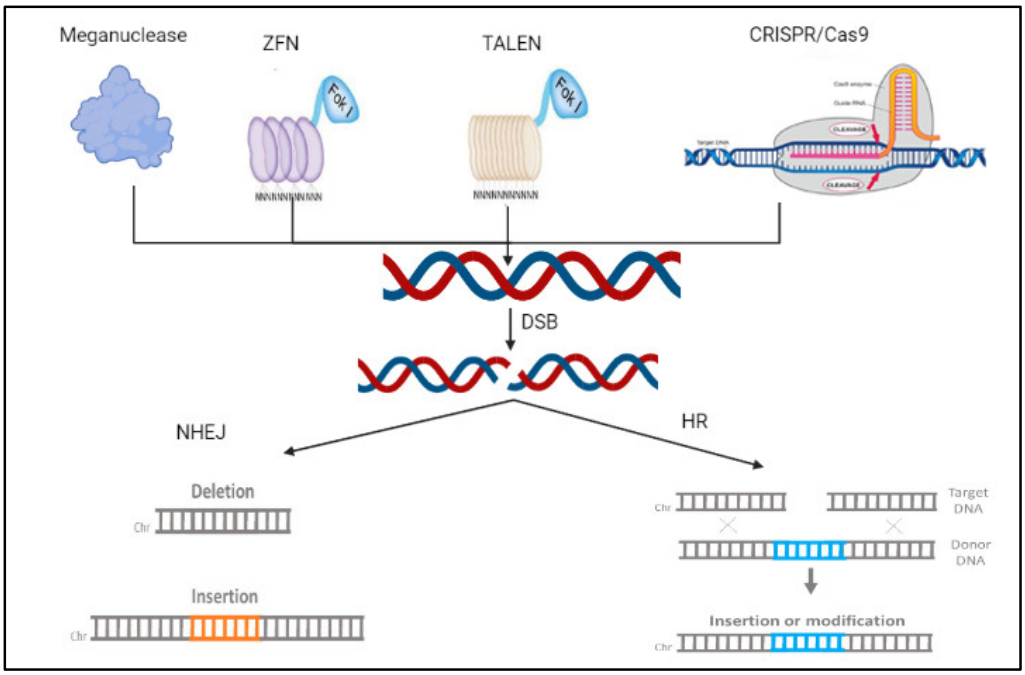

Figure 1. Schematic illustration of the different generations of nucleases used for genome editing and the DNA repair pathways used to modify target DNA

The fourth and most recent GET is based on Streptococcus pyogenes' adaptive immune system, which uses CRISPRs to express DNA fragments (spacers) taken from invading pathogens in order to neutralize previously encountered invasive DNA sequences (Marraffini and Sontheimer 2010). A designed guide RNA (gRNA) directs the Cas9 endonuclease to the desired nucleotide site on the genome. A protospacer adjacent motif (PAM) sequence at this position must also be recognised by the Cas9 enzyme for a DSB to be introduced in the target region (Jinek et al., 2012). The DSBs caused by various GE approaches are predominantly repaired by the processes of homology directed repair (HDR) and non-homologous end joining (NHEJ) (Symington and Gautier 2011). Small insertion or deletion (InDel) mutations are considerably introduced via NHEJ at the cut location, which results for example in the gene silencing.

When compared to creating protein motifs for ZFNs or TALENs, generating gRNAs for CRISPR/Cas9-mediated GE is substantially less expensive, higher efficient and relies on protein engineering for its improvement and development (Christian et al., 2010; Bortesi and Fischer, 2015). Because of its simplicity, precision, specificity, adaptability, capacity to multiplex characteristics, low risk of off-target effects and ease of use, CRISPR/Cas9-based genome editing has quickly taken the lead as the most popular genetic engineering strategy for creating superior crop varieties (Yin et al., 2017). So long as sequence information for the target gene is available, CRISPR/Cas9 systems can be successfully used on any crop to produce high yields under biotic or abiotic stress (Yin et al., 2017). Numerous examples of targeted mutagenesis in agricultural plants, including gene knockouts, alterations, and the activation and repression of target genes, have increased the application of the CRISPR/Cas9 system extremely quickly in recent years (Abdelrahmana et al., 2018). There are many different cereal crops have been modified utilizing the CRISPR/Cas9 system (Hillary and Ceasar, 2019).

How are edited plants produced?

There are five critical steps to construct gRNAs for a CRISPR/Cas9 GE application in any crop:

Gene target identification and guide RNA (gRNA) designing

To construct gRNAs for a CRISPR/Cas9 GE application in any crop, there are two essential steps: selecting a genomic target site and developing a complementary guide sequence. The target gene's sequence information must be accessible in order to obtain a genomic target, and a complete genome is preferred to evaluate off-target activities. The available sequenced genomes, which include many cereal crops, can be interactively accessed through genome browsers like Phytozome (https://phytozome.jgi.doe.gov/pz/portal.html), NCBI (https://www.ncbi.nlm.nih.gov) and Ensembl Plants (https://plants.ensembl.org/index.html). More sequenced and annotated cereal crop genomes have become available to the public as genome sequencing techniques have developed and become more affordable. But for all crops, gene target identification continues to be a very important step. Potential genes of interest can be discovered using gene ontologies from related species, previous studies, or well used pathways. The method used and the desired phenotype determine the precise placement of the target area within a given genome. The gRNA target sequence should be found in an exonic, translated region within the first half of the gene for functional gene knockdown (Kaur *et al*., 2020). To alter gene expression, gRNAs may target a target gene's promoter region or upstream ORF (Kaur *et al*., 2020).

In molecular biology, bioinformatics has emerged as a crucial method for GE. The two primary criteria for CRISPR/Cas9 GE are efficacy and specificity. Efficiency is a measure of how well a sgRNA targets a particular sequence and directs the Cas9 enzyme to edit the targeted sequences; it is typically expressed as the proportion of modified cells. Specificity refers to whether or not CRISPR/Cas9 editing events are one-of-a-kind and whether or not they have unintended side effects. sgRNA design has taken into account a variety of variables that affect the effectiveness and specificity of CRISPR/Cas9 GE (Naeem and Alkhnbashi, 2023; Chuai *et al*., 2018). The RNP complex's affinity for the targeted DNA sequences is established by the hybridization of sgRNAs and DNA sequences through sequence complementarily. According to earlier research, differing binding sites cause significant variations in cleavage efficiency and specificity between various organisms (Tang *et al*., 2019). A number of web-accessible databases have been created related to gRNA design, finding off-target sites, CRISPR systems and databases (Table 1). Based on the analysis, these databases not only offer useful resources for sgRNA selection but also highlight the critical elements that influence sgRNA specificity and efficacy, making it easier to continue optimizing sgRNA design (Alipanahi *et al*., 2023; Naeem and Alkhnbashi, 2023).

Construct design and transformation

Cloning and transformation of plasmid constructs containing the elements to express Cas9 nucleases and gRNAs are necessary for GE in agricultural plants after gene identification and gRNA design. The type of intended sequence modifications will determine the Cas9 nuclease that is cloned. While Cas9 is expressed via CaMV35S, RNA polymerase (Pol) II or species-specific ubiquitin promoters, gRNA is expressed under Pol III promoters, U3 and U6 (Hahn et al., 2020). SnapGene and Benchling are two online tools that can be used to support build design (*www.snapgene.com* and *www.benchling.com*). The intricacy and redundancy of the plant genome make it likely that changing one mutation won't be enough to affect cellular activities. Through the integration of numerous promoter-designed gRNA cassettes into a single vector system, The CRISPR/Cas9 system may modify multiple genes (Hyun, 2020; Abdallah *et al*., 2022). To assure construct activity, these

constructs must undergo experimental validation. Stable transformation enables long-lasting gene alterations that produce heritable alleles (Altpeter *et al*., 2016).

Table 1. Computational tools and resources related to gRNA design, finding off-target sites, CRISPR systems and databases

Tool/ software	Major feature	Database or Website	Reference
CRISPOR	Creating, assessing, and cloning CRISPR/Cas9 system guide sequences Giving primers to build vectors; displaying the mismatch number; and linking the genome browser to off-target	https://crispor.tefor.net/	Haeussler *et al*., 2016
CHOPCHOP	Generating primers and displaying the chromosomal site	https://chopchop.cbu.uib.no/	Labun *et al*., 2019
CRISPR RGEN Tools	Providing a variety of predictive models that are standalone, downloadable, and capable of forecasting potential off-target numbers	https://www.rgenome.net/Cas-designer/	Park *et al*., 2015
E-CRISP	Genomics-scale libraries that can be downloaded and regularly updated	https://www.e-crisp.org/ECRISP/index.html	Heigwer *et al*., 2013
CRISPRscan	creating tracks for a genome browser, designing sgRNAs for specific gene sites, and scanning the entire genome for off-target effects	https://www.crisprscan.org/	Moreno-Mateo *et al*., 2015
CCTop	Identifying mismatches and forecasting off-target effects, predicting sgRNA efficiency	https://cctop.cos.uniheidelberg.de/	Stemmer *et al*., 2015
CRISTA	Providing a framework for machine learning, identifying off-targets, and evaluating targets	https://crista.tau.ac.il/	Abadi *et al*., 2017
CRISPR-GE	creating vectors and designing primers for on-target amplification	https://skl.scau.edu.cn/	Xie *et al*., 2014
CRISPR-P	gRNA sequence analysis, as well as on-target and off-target screening	https://crispr.hzau.edu.cn/CRISPR2/	Liu *et al*., 2017
CRISPR-Local	Creates sgRNAs in plants and other species	http://crispr.hzau.edu.cn/CRISPR-Local/	Sun *et al*., 2019
CRSeek	It locates both on- and off-target websites.	https://github.com/DamLabResources/crseek	Dampier *et al*., 2018
CRISPResso	Potential is found both on and off-targets	http://github.com/lucapinello/CRISPResso.	Pinello *et al*., 2015
Cas-OFFinder	It looks for probable off-target places and displays information about their locations, directions, and number of mismatches.	http://www.rgenome.net/Cas-offinder	Bae *et al*., 2014
CasOT	With user-specified PAM types, mismatch rates, and genomes, it identifies potential off-target locations	http://eendb.zfgenetics.org/Casot/	Xiao *et al*., 2014
CRISPRitz	The potential implications of putative off-target sequences on the functioning genome are enumerated, annotated, and assessed	https://github.com/pinellolab/CRISPRitz	Cancellieri *et al*., 2020
CRISPRidentify	It finds all potential CRISPR arrays.	https://github.com/BackofenLab/CRISPRidentify	Mitrofanov *et al*., 2021
CRISPRloci	Definition of each locus's CRISPR leaders; prediction of the direction of each CRISPR array; annotation of the Cas9 genes	https://rna.informatik.unifreiburg.deCRISPRloci	Alkhnbashi *et al*., 2021

CRISPR-DAV	A high-throughput workflow for the CRISPR-NGS data analysis	https://github.com/pinetree1/crispr-dav.gitand https://hub.docker.com/r/pinetree1/crispr-dav	Wang *et al*., 2017
CRISPRdigger	Improving the accuracy of a query genome	http://www.healthinformaticslab.org/supp/	Ge *et al*., 2016
BATCH-GE	It recognizes and records precise genome editing events, such as indel mutations, and estimates the relevant mutagenesis efficiencies.	https://github.com/WouterSteyaert/BATCH-GE.git	Boel *et al*., 2016
CRISPR-GA	It provides estimations for the HDR and NHEJ as well as a detailed report on the location and properties of the indels.	http://crispr-ga.net/documentation.html	Güell *et al*., 2014
CRISPR Plant v2	For extremely specialised sgRNAs	https://www.ncbi.nlm.nih.gov/pmc/articles/PMC6330547/	Minkenberg *et al*., 2019
CRISPRlnc	SgRNA manual database	https://www.crisprlnc.org	Chen *et al*., 2019
Cpf1-Database	Tool for Cpf1 design	http://www.rgenome.net/cpf1-database/	Park and Bae, 2018
CrisprGE	Cas9-Central repository for CRISPR	http://crdd.osdd.net/servers/crisprge/	Kaur *et al*., 2016

Agrobacterium-mediated transformation and biolistic bombardment are two of the most widely used stable transformation techniques in plants. Following plant transformation, it's crucial to promote callus growth, differentiation, and regeneration into shoot and root tissue (Altpeter *et al*., 2016; Do *et al*., 2018). In the majority of cereal transformation techniques, an immature embryo is used as the transformable explant, and plasmid administration may be accomplished via either biolistic bombardment (Liu *et al*., 2019) or *Agrobacterium* (Do *et al*., 2018) (Figure 2). Notably, biolistic bombardment can transport CRISPR/Cas9 as ribonucleoprotein (RNP), as has been shown in significant crops like maize (Svitashev *et al*., 2016) and wheat (Zhang *et al*., 2016). This method avoids bacterial cloning, crossover, or segregation, but it is very expensive to remove constructed transgenes from modified plants. By subjecting regenerates to a selective antibiotic or herbicide for which a resistance gene is present in the GE construct, likely transgenic plants are continuously screened during the regeneration process.

Mutation screening

Barcode sequencing, a Cleaved Amplified Polymorphic Sequence (CAPS) screening technique that detects mismatches in DNA strands during PCR (Mohr *et al*., 2022), (Smith *et al*., 2006), or RFLP can all be used to confirm gene edits in primary transformants if the gene edit breaks the enzyme restriction digestion site. In order to validate gene changes, next-generation or Sanger sequencing should be used (Liu *et al*., 2021) (Figure 2). These methods can pinpoint the precise nature of gene alterations. It should be noted that in order for regulatory agencies to accept gene-edited crops as a molecular breeding technique, they must undergo several generations of outcrossing to guarantee that editing DNA sequence is no longer present (Shinwari *et al*., 2018).

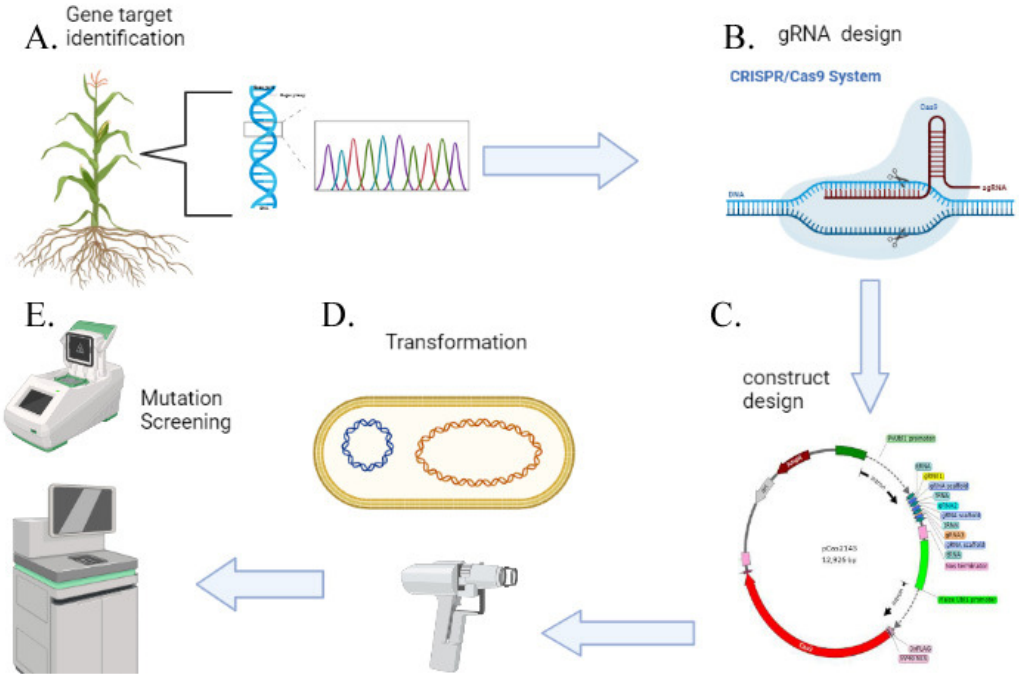

Figure 2. The different steps for creating genome editing plants. A: gene target identification using genome sequencing. B: gRNA design using bioinformatics tools. C: construct design using expression vector have Cas99 gene and gRNA sequences. D: Vector transformation into plant cell via transformation methods such as *Agrobacterium*-mediated transformation or biolistic bombardment. E: mutation conformation and screening using CAPS PCR or DNA sequencing

Cereal genome editing

Due to their widespread importance for human nutrition, cereal crops have been the main targets for genetic modification. For a very long time, it was challenging to genetically modify many of these essential cereals, mostly due to their inherent constraints related to their resistance to Agrobacterium infection and their resistance to in vitro regeneration (Shrawat and Lörz, 2006). Cereal genomes vary widely in terms of size, complexity, and sequence features, allowing researchers to compare different species to pinpoint nuclease- and species-dependent variables influencing the results of genome editing. This strategy could make it easier to develop future genome editing tactics (Zhu *et al*., 2017). Recently, scientists' interest in genetically edited plants has increased, and according to the research in the PubMed database, rice was the most researched plant, followed by wheat and maize (Figure 3). In cereal crops like rice and maize, as well as a smaller number of studies in barley, wheat and sorghum, genome editing techniques are widely used.

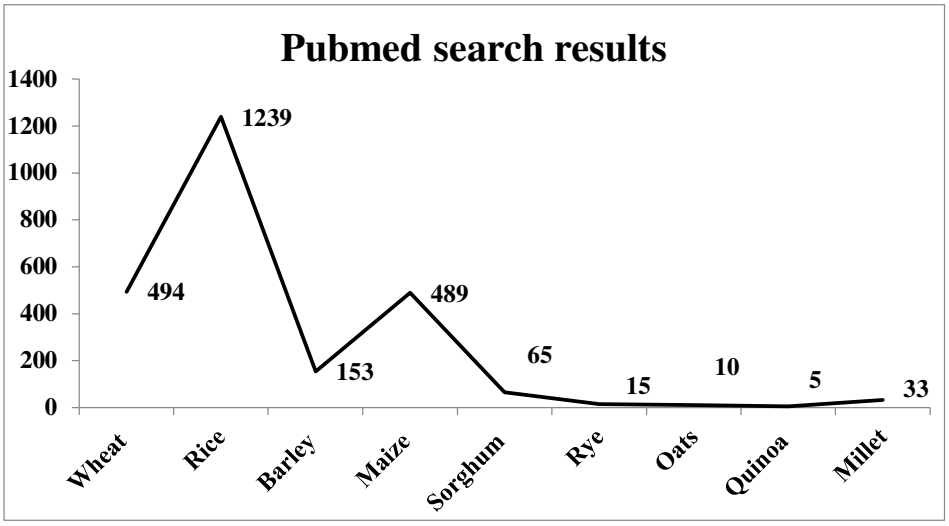

Figure 3. The research results on the PubMed database for cereal crop genome editing on November 2023

The new GE techniques

A relatively recent technique for modifying the genome, base editing (BE) is derived from CRISPR-Cas9. Base editors (BEs) do not cause double-stranded breaks in the genome, in contrast to conventional CRISPR systems. Base editing techniques are based on "catalytically dead" Cas9 (dCas9), which is coupled to bacterial enzymes termed DNA deaminases and is incapable of cleaving DNA (Porto *et al*., 2020). This technology was developed by David Liu's lab at the Broad Institute. Adenine deaminases, which cause A to G substitutions, were developed from bacterial enzymes expressly for base editing applications, whereas cytotidine deaminases, which produce C to T substitutions, are found naturally in bacteria. Researchers can induce substitutions in DNA by fusing dCas9 to an adenine deaminase (ABEs) or cytidine deaminase (CBEs) and giving a sgRNA to direct it to the target sequence (Porto *et al*., 2020). Prime editing (PE) is a recently established precise genome editing technique that allows for desired tiny deletions, insertions, and base conversions of any kind. Prime editing gRNA (pegRNA) and a prime editor protein make up PEs. Reverse transcriptase, which may be directed to the genomic locus via pegRNA, is fused with an engineered Cas9 nickase (H840A) to create the PE protein (Scholefield and Harrison, 2021; Anzalone *et al*., 2019). In order to start reverse transcription, the pegRNA combines a primer binding site, a reverse transcriptase template encoding the necessary modifications, and a gRNA that recognises the target genomic region (Liu *et al*., 2023; Anzalone *et al*., 2019).

Targeted genome editing in rice

Rice (*Oryza sativa* L.), one of the most important food crops in the world, feeds about 50% of the world's population. Early in the twenty-first century, there has been a rapid advancement in our understanding of the molecular mechanisms that control development and morphogenesis in rice (Hirano *et al*., 2014; Tanaka *et al*., 2014). According to Jia (2003), conventional rice breeding requires ten years to backcross and cross several blast-resistant genes into a rice variety. One of the most crucial quantitative characteristics in the production of rice is grain weight.

There are many previous studies that used gene editing to develop editing rice plants that were tolerant to both abiotic and biotic stresses. In particular, salinity and drought stresses are major constraints in reducing the yield and quality of rice. Many genes were targeted by different genome editing tools to generate editing

rice plants tolerant to salinity and drought such as *OsPDS, OsDERF1, SRL1, SRL2, OsAAA-1, OsAAA-2, OsNAC006, OsAOX1a, OsDST, OsERA1, OsPYL9, OsNAC14* and *OST2* (Table 2). For example, CRISPR-Cas9 was used to target *OsMIR408* and *OsMIR528* genes as well as *miR815a/b/c* and *miR820a/b/c* genes to change in the expression profiles. In mature miRNA regions, they produced single-base pair (bp) indels alterations. It was discovered that sizable deletions at either the mature miRNA or the corresponding miRNA quickly eliminated miRNA function. They found that *OsMIR528* was a positive regulator of salt stress.

Table 2. Examples for genome editing application in rice

Genes	Traits studied	System & strategy	References
OsMYB	Gene knockout	CRISPR	Miao *et al*., 2013
OsPDS and *OsBEL*	Targeted mutagenesis	CRISPR	Xu *et al*., 2016
OsCSA, OsPMS3, OsDERF1, OsGN1a, OsTAD1, OsMST7 and *OsMST8*	Targeted mutagenesis	TALEN	Zhang *et al*., 2016
Os11N3	Gene editing	TALEN	Li *et al*., 2012
OST2	Salt tolerance	CRISPR	Osakabe *et al*., 2016
OsPDS	Drought tolerance	CRISPR	Shan *et al*., 2013
OsGSTU, OsMRP15 and *OsAnP*	metabolism	CRISPR	Ma *et al*., 2015
OsWaxy	metabolism	CRISPR	Ma *et al*., 2015
ALS1	Herbicide tolerance	CRISPR	Sun *et al*., 2016
ESPS	Herbicide tolerance	CRISPR	Li *et al*., 2016
Hd2, Hd4 and *Hd5*	Development	CRISPR	Li *et al*., 2017
GW2, GW5 and *TGW6*	Development	CRISPR	Xu *et al*., 2016
OsSWEET13	Loss of function	CRISPR	Zhou *et al*., 2017
MPK1, MPK2, MPK5 and *MPK6*	Development	CRISPR	Minkenberg *et al*., 2017
Annexin	Loss of function cold	CRISPR	Shen *et al*., 2020
SNF 1-related protein kinase 2	Loss of function cold	CRISPR	Lou *et al*., 2017
*NRT1.1.B*and*SLR1*	base editing	CRISPR	Lu and Zhu, 2017
OsMIR408 and *OsMIR528*	New insights into MicroRNA function	CRISPR	Zhou *et al*., 2017
OsSWEET11 and*OsSWEET14*	Bacterial resistance	CRISPR	Jiang *et al*., 2013
OsERF922	Blast disease	CRISPR	Wang *et al*., 2016
OsBADH2, OsDEP1, OsSD1 and *OsCKX2*	Fragrance	TALEN	Shan *et al*., 2013
Os11N3	Bacterial resistance	TALEN	Li *et al*., 2012
OsCSA	Photoperiod sensitive male sterility	TALEN	Zhang *et al*., 2016
OsDERF1	Drought tolerance	TALEN	Zhang *et al*., 2016
OsAnn3	Cold tolerance	CRISPR	Shen *et al*., 2017
OsAnn5	Cold tolerance	CRISPR	Shen *et al*., 2017
*SRL1*and *SRL2*	Drought tolerance	CRISPR	Samai *et al*., 2015
*OsAAA-1*and *OsAAA-2*	Drought tolerance	CRISPR	Okada *et al*., 2019
OsNAC006 (transcription factor)	Drought and heat tolerance	CRISPR	Chen *et al*., 2021
OsAOX1a	Drought resistance	CRISPR	Osawa *et al*., 2015
OsDST	Salinity and drought tolerance	CRISPR	Moya *et al*., 2021

OsERA1 and *OsPYL9*	Drought tolerance	CRISPR	Jia *et al*., 2019
SAPK2	Salinity and drought tolerance	CRISPR	Yan *et al*., 2019
OsPMS3	Male-sterile	CRISPR	Osawa *et al*., 2015
Csa	Photosensitive-genic	CRISPR	Schneider *et al*., 2023
TMS5	Temperature tolerance	CRISPR	Wang *et al*., 2019
OsNAC14	Drought tolerance	CRISPR	Demirer *et al*., 2019
OsPUB67	Drought tolerance	CRISPR	Demirer *et al*., 2021
OsbHLH024	Salinity	CRISPR	Alam *et al*., 2022
OsRR22	Salinity	CRISPR	Zhang *et al*., 2019
*OsRAV2, OsNAC041*and *OsmiR535*	Salinity	CRISPR	Shelake *et al*., 2022
*OsRR9*and *OsRR10*	Salinity	CRISPR	Wu *et al*., 2019
OsNAC041	Salinity	CRISPR	Kumar *et al*., 2023
OsOTS1	Salinity	CRISPR	Erdoğan *et al*., 2023
OsDST	Drought and salinity tolerance	CRISPR	Santosh *et al*., 2020
SAPK2	Salinity tolerance	CRISPR	Erdoğan *et al*., 2023
C287T	Herbicide tolerance	CRISPR	Wang *et al*., 2022
BEL	Herbicide tolerance	CRISPR	Naik *et al*., 2022
OsALS1	Herbicide tolerance	CRISPR	Kuang *et al*., 2020
EPSPS	Herbicide tolerance	CRISPR	Sony *et al*., 2023
SF3B1	Herbicide tolerance	CRISPR	Serrat *et al*., 2019
OsTubA2	Base editing	CRISPR	Wang *et al*., 2022
OsHAK1	Low cesium accumulation	CRISPR	Kumar *et al*., 2023
OsPRX2	Potassium deficiency tolerance	CRISPR	Wang *et al*., 2022
OsARM1	Arsenic tolerance	CRISPR	Wang *et al*., 2022
OsLCT1	Cadmium tolerance	CRISPR	Wang *et al*., 2022

In addition, GET was used to develop biotic resistance rice including induction indel mutations in many genes such as *OsMYB, OsPDS, OsBEL, OsCSA, OsPMS3, OsDERF1, OsGN1a, OsTAD1, OsMST7, OsMST8,* and *Os11N3* (Table 2).

Also, this technology was used in editing genes controlling many development stages in rice plants. The construct gDL-1 was first introduced by Ikeda *et al*. (2016). It created a guide RNA that was directed at the *DL* (*DROOPING LEAF*) gene. *DL* controls the development of the midrib in the leaf and the carpel specification in the flower. The result of gene disruption should be obvious since the drooping leaf phenotype in regenerated seedlings is caused by the absence of *DL* function. Seven out of the nine plants evaluated as transgenic plants harbouring gDL-1 showed bi-allelic mutations, demonstrating the remarkable efficiency with which the *DL* gene was disrupted. Therefore, CRISPR-Cas9 technology can be a practical and effective tool in studies of rice developmental processes.

Wheat genome editing

One of the most extensively produced crops in the world and an essential food source is wheat. It is one of the most significant sources of plant protein and contributes an average of 19% of the total calories in human diet. The manufacturing of leavened bread and a number of other foods is made possible by the gluten found in bread wheat, which is unique among cereal crops (Shewry, 2009). Since wheat is a vital commodity with a supply-demand imbalance, it is crucial to improve production generally, including in regions with less-than-ideal conditions such as water scarcity, salt, and high temperatures. The creation of crop cultivars with better drought tolerance is a prominent objective for wheat improvement programs as irrigation water supplies have become scarcer. Due to the overall warming brought on by global climate change as well as the increased frequency of droughts and other abiotic stresses, which can cut agricultural yields by as much as 50%, these factors pose a serious danger to the food security (Reynolds *et al*., 2021).

A challenging crop for genomics studies has been wheat because of its huge genome (16000 Mb). Prior estimates placed the number of genes in the bread wheat genome at over 128,000 (Montenegro *et al*., 2017), with about 80% of the genome being made up of repetitive DNA sequences (Bhalla *et al*., 2017). Bread wheat has three subgenomes (A, B, and D with x = 7 and n = 21) that are controlled into seven homoeologous groups at the cytogenetic level. Although there has always been disagreement over the progenitor of the B genome, the diploid progenitors of the A, B, and D subgenomes have been found (Gill *et al*., 2004). However, because bread wheat has a big genome and three closely linked subgenomes, investigations in the field of molecular cytogenetics have proven challenging (Gill *et al*., 2004). Several attempts have been made in recent years to create new tools for wheat genomics research. Gene targeting systems, physical and genetic molecular maps and expression sequences tags (EST) collections are a few of them (Feuillet and Keller, 2002). Comparative genomics and functional genomics research have been utilized to develop crop improvement programs as well as examine evolutionary links in a variety of important wheat varieties (Jordan *et al*., 2007). This made it possible to create sets of candidate genes for specific traits, which can be used to understand their biology (Bagge *et al*., 2007).

A feature of the CRISPR/Cas9 system is multiplexing, or the simultaneous targeting of several genes with a single genetic construct. Multiple sgRNAs can be introduced as polycistronic forms as well as separate transcription units (Čermák *et al*., 2017). Many experiments have been reported in bread wheat using different explants with different transformation methods, such as electroporated microspores (Bhowmik *et al*., 2018), PEG-transfected protoplasts (Zhang *et al*., 2017), cell suspension Agrobacterium transformation (Upadhyay *et al*., 2013), immature embryos and immature embryo Agrobacterium transformation (Howells *et al*., 2018; Singh *et al*., 2018), immature embryos, immature embryo particle bombardment transformation (Gil-Humanes *et al*., 2017; Hamada *et al*., 2018). Multiplexing, or the simultaneous targeting of many genes with a single molecular construct, is one of the added benefits of the CRISPR/Cas9 system. Both individual transcription units and polycistronic forms of multiple sgRNAs can be inserted (Čermák *et al*., 2017; Rezk *et al*., 2023; Sattar *et al*., 2023). Many experiments have been reported in bread wheat using different explants with different transformation methods, such as electroporated microspores (Bhowmik *et al*., 2018), PEG-transfected protoplasts (Liang *et al*., 2017; Zhang *et al*., 2017), cell suspension Agrobacterium transformation (Upadhyay *et al*., 2013), immature embryos and immature embryo *Agrobacterium* transformation (Howells *et al*., 2018; Singh *et al*., 2018), immature embryos, immature embryo particle bombardment transformation (Gil-Humanes *et al*., 2017; Wang *et al*., 2018; Hamada *et al*., 2018). Additionally, wheat's hexaploid nature and gene functional redundancy make the genetic improvement process difficult and time-consuming (Li *et al*., 2020). It is crucial to increase the flexibility of wheat production through the application of cutting-edge technologies in order to assure global food and ecological security (Li *et al*., 2021).

To maintain long-term food security, the creation of wheat cultivars resistant to different biotic and abiotic stress is a significant problem (Pandey *et al*., 2022). Genome editing may be the solution to genetic improvement for plant species with complicated genetic structures, such as wheat (Pandey *et al*., 2022, El-Beltagi *et al*., 2023). Many genes were editing for salt and drought tolerant such as *Sal, TaDREB2, TaERF3, TaMLO-B1, TaHAG1* and *ALS* and for biotic stress such as *EDR1, TaMLO-A1, SIMlo1* and *TaMLO-D1* (Table 3). *TaMLO*, a locus for resistance to powdery mildew, was modified in wheat in the first successful CRISPR/Cas9 experiment. Significant losses in wheat yield are brought on by *Blumeria graminis* f. sp. tritici's powdery mildew infections, and knocking down the TaMLO makes the disease resistant (Shan *et al*., 2013). Wang *et al*. (2014) also provided details on altering the *TaMLO*-Aallele using CRISPR/Cas9 technology. The regenerated *TaMLO*-edited wheat had a 5.6% mutation frequency. More recently, Zhang *et al*. (2016) developed TaEDR wheat lines utilizing CRISPR/Cas9 technology by knocking down the three homologs of wheat TaEDR, for powdery mildew resistance.

Table 3. Genome editing application in wheat

Genes	Traits studied	System &strategy	References
α-gliadin	Development	CRISPR	Sánchez-León *et al*., 2018
Three *EDR1*	Disease resistance	CRISPR	Zhang *et al*., 2017
Sal	Salt tolerant	CRISPR	Abdallah *et al*., 2022
SIMlo1	Mildew resistance	CRISPR	Nekrasov *et al*., 2013
TaMLO-A1	Mildew resistance	CRISPR	Wang *et al*., 2014
TaMLO-A1	Powdery mildew resistance	TALEN	Wang *et al*., 2014
TaMLO-B1	Powdery mildew resistance	TALEN	Wang *et al*., 2014
TaMLO-D1	Powdery mildew resistance	TALEN	Wang *et al*., 2014
TaDREB2 and*TaERF3*	Drought tolerance	CRISPR	Li *et al*., 2023
TaHAG1	Salt tolerance	CRISPR	Xiang *et al*., 2019
ALS	Herbicide tolerance	CRISPR	Li *et al*., 2023
TaGW7	Grain shape	CRISPR	Wang *et al*., 2019
TaGW2	Grain size	CRISPR	Wang *et al*., 2019

Targeted genome editing in barley

Malted barley (*Hordeum vulgare*) is the world's fourth-largest cereal crop. Barley is grown in temperate regions of the world because it is hardy and adaptable. It has numerous uses in the brewing, distilling and malting sectors as well as animal feed. Although barley today only makes up a small portion of the average person's diet, it nevertheless serves as a major source of dietary fiber and b-glucan and is still a common meal in many countries, including Tibet. The success in increasing barley's yield potential is unsatisfactory given the continuously increasing demand for food production, much like that of the other most important cereal crop species. The discovery and use of new genes that regulate grain size and grain number, the two primary factors in yield in cereals, may help to solve this issue (Han *et al*., 2020).

A natural model for the genetics and genomics of the barley (*Triticeae tribe*), is a diploid member of the grass family Han *et al*. (2020). Barley's genome has been successfully edited using CRISPR/Cas9-based methods (Gasparis *et al*., 2018; Holubova *et al*., 2018). As in rice and wheat, genome editing technology was utilized for editing different genes in barley such as *HvPM19, HvITPK1* and *ALBOSTRIANS* for salt tolerant and development process (Table 4).

Lawrenson *et al*. (2015) show how to employ RNA-guided Cas9 to cause specific mutations in barley, and they reveal that the changes are durable across generations. They demonstrated the applicability of this method for quick investigations of gene function by demonstrating that knockout phenotypes can be recovered as early as the first T0 generation. In order to promote the potential for later biotechnological applications, they generated transgene-free barley plants with stably inherited mutations in the target gene. The traits shown here make RNA-guided Cas9 particularly useful for functional investigations in these species, as crop genomes frequently contain numerous closely related sequences. Han *et al*. (2020) developed a different culture-based strategy to effectively grow transgenic and GE plants from commercial barley varieties. The *HvPDS* gene was targeted via *Agrobacterium*-mediated transformation on microspore-derived callus, and t0 albinos with specific mutations were effectively obtained from commercial varieties. Three targets were further edited, with an average mutation rate among the five kinds being 53%. With varying mutation rates among targets and types, Cas9 caused a significant part (69%) of single-baseindels and two-base deletions in the target sites in 51 examined T0 individuals. It demonstrates potential for functional genomics and the use of CRISPR technology for precision commercial variety improvement. Gasparis et al. (2019) utilized an RNA-guided Cas9 method to produce the *ckx1* and *ckx3* mutant lines with knockout mutations in the *HvCKX1* and *HvCKX3* genes, respectively, to investigate the possibility of increasing the grain yield of barley by removing *CKX* genes. Then, homozygous, transgene-free mutant lines were chosen and examined. When CKX enzyme activity was measured in the spikes of the *ckx1* and *ckx3* lines, it was found to have significantly decreased from that of the control plants. Using RNA-guided Cas9, Yang et al. (2020) was able to successfully eliminate the D-hordein portion of the barley storage protein. At two distinct target locations, mutation frequencies of 25% and 14% were recorded. In the T1 generation, homozygous mutant plants without T-DNA were discovered. When compared to the parent plant and the control non-edited line, barley grains from T2 seeds lacking D-hordein proteins had considerably smaller grains.

Table 4. Genome editing application in barley

Genes	Traits studied	System & strategy	References
HvPM19	Hormone signalling	CRISPR	Lawrenson *et al*., 2015
HvPM19	Seed dormancy	CRISPR	Lawrenson *et al*., 2015
HvPAPhy	Phytase reduction and seed development	TALEN	Wendt *et al*., 2013
HvITPK1	Salinity tolerance	CRISPR	Borrelli *et al*., 2018
HvGBSSIa	Low amylose content	CRISPR	Liu *et al*., 2021
ALBOSTRIANS	chloroplast development	CRISPR	Li *et al*., 2021
CMF7	chloroplast development	CRISPR	Li *et al*., 2021
ASY1	meiotic	CRISPR	Li *et al*., 2023
MUS81	meiotic crossover	CRISPR	Marini *et al*., 2023
ZYP1	transverse filament protein	CRISPR	Liu *et al*., 2021

Targeted genome editing in maize

There is widespread knowledge of and growing significance of maize (*Zea mays* L.) to global agriculture, the global economy, and food security. One of the most significant crops in the world, maize, is widely utilized as food, fuel, feed, and raw material by many industries (Andorf *et al*., 2019). The yields of maize have historically been significantly impacted by fluctuations in temperature, salt, drought, precipitation, and their interactions (Ray *et al*., 2015; Daryanto *et al*., 2016). Yield and abiotic stress tolerance are complicated variables that are frequently subject to strong environmental effect and linked to genetic regions with minor effects.

Recent and quick technology advancements in genome sequencing, computational biology, and new genotyping and phenotyping technologies have been substantially incorporated into current maize breeding programs. Technology advancements like twofold haploid technology and genome sequencing are deeply included into maize breeding programs (Andorf *et al*., 2019).

The majority of GE studies in maize have been carried out utilizing genetic transformation protocols (*Agrobacterium tumefaciens* or biolistic) (Lorenzo *et al*., 2023; Kausch *et al*., 2021; Yassitepe *et al*., 2021). Many genes were editing in maize using different GET including *IPK1, Rht-B1, Rht-D1, LIG, ALS2, MS26* and *MS45* (Table 5). Shukla *et al*. (2009) demonstrated that simultaneous ZFN expression and delivery of a straightforward heterologous donor molecule results in the targeted insertion of an herbicide-tolerance gene at the specified locus. These genetic alterations are faithfully passed down to subsequent generations by ZFN transgenic maize plants. Herbicide resistance and the anticipated change in the inositol phosphate profile in growing seeds are both produced by insertional disruption of one target gene, *IPK1*.

Table 5. Genome editing application in maize

Genes	Traits studied	System & strategy	References
Auxin regulated genes	Drought tolerance	CRISPR	Shi *et al*., 2017
IPK1	Herbicide resistance	ZFN	Shukla, 2009
ZmAgo18a and *ZmAgo18b*	targeted mutagenesis	CRISPR	Char *et al*., 2015
ZmCENH3	base editing	CRISPR	Zong *et al*., 2017
LIG, ALS2, MS26 and *MS45*	Herbicide tolerant	CRISPR	Svitashev *et al*., 2016
PAT	Herbicide tolerant	ZFNs	Schornack *et al*., 2006
ZmIPK1	Herbicide tolerant	ZFNs	Shukla *et al*., 2009
ZmTLP	Trait stacking	ZFNs	Ainley *et al*., 2013
ZmPDS, ZmIPK1A, ZmIPK and *ZmMRP4*	Biosynthesis	TALEN	Liang, *et al*., 2014
MS26	Male sterile	MNs	Djukanovic *et al*., 2013
ZmHKT1	Salinity tolerance	CRISPR	Zhang *et al*., 2018
ALS1 and *ALS2*	Herbicide tolerance	CRISPR	Kuang *et al*., 2020
MS26	Herbicide tolerance	CRISPR	Kuang *et al*., 2020
Psy1	Seed color	CRISPR	Zhu *et al*., 2016
SH2	Super sweet	CRISPR	Dong *et al*., 2019
GBSS	Waxy corn	CRISPR	Dong *et al*., 2019
Wx1	Waxy corn	CRISPR	Gao *et al*., 2020

Targeted genome editing in sorghum

Sorghum bicolor (L.), sorghum, is a significant annual C4 cereal crop with special qualities that make it useful for practically all renewable energy and green technology applications. Currently, sweet sorghums are utilized in a sugar-to-ethanol process in China, the Philippines, and India while the grain is used as a feedstock in the grain-to-ethanol process in the United States. Sorghums with high biomass yields are being researched for both lignocellulosic and cellulosic renewable energy applications. Sorghum is being investigated by other nations as a possible supply of high-value C molecules for the production of renewable fuels and other significant industrial chemicals, as well as a renewable building material. Sorghum has the potential to produce crops with high yields while using minimal water and other resources, has strong research capabilities, a thriving seed market, and a long history of research on production and cultural practices (Dahlberg, 2019). Sorghum may be produced in dry and semiarid areas where maize cannot since it is a drought-tolerant crop. In many

parts of the world, sorghum grain is a significant staple diet for people and a source of feed for cattle. The grain of sorghum lacks critical amino acids like Lys, and its protein is hard to digest, similar to maize (Aboubacar *et al*., 2001).

GET have been used for modified different genes in sorghum for biotic, abiotic and developmental stages including *K1C, LGS1, SbPRR37, Ma2* and *FT* genes (Table 6). Li *et al*. (2018) targeted the *k1C* genes using a CRISPR/Cas9 gene editing technique. *Cinnamyl alcohol dehydrogenase (CAD) and phytoenedesaturase (PDS)*, two target genes that were altered by CRISPR/Cas9 by bombardment, were examined by Liu *et al*. (2019). The sorghum genotype Tx430 has experienced successful genome editing. Additionally, it has been demonstrated that CRISPR/Cas9 successfully altered the target gene in sorghum by sequencing the PCR product of transgenic plants. The outcomes showed that the altered gene had been handed down to the following generation. More research is being done, including enhancing the promoters for guide RNA (gRNA) and Cas9 in sorghum.

Table 6. Genome editing application in sorghum

Genes	Traits studied or Effect	System & strategy	References
*K1C*genes	Development	CRISPR	Li *et al*., 2018
Sb-CENH3	Induces haploid sorghum	CRISPR	Che *et al*., 2018
α-Kafirin	Reduced α-kafirin levels	CRISPR	Li *et al*., 2018
LGS1	Parasite resistance	CRISPR	Bellis *et al*., 2020
SbPRR37	Early flowering phenotypes	CRISPR	Baloch *et al*., 2023
Ma2	Early flowering phenotypes	CRISPR	Liu *et al*., 2021
FT genes	Flowering time	CRISPR	Lee, 2023
TIL1	Vegetative branching	CRISPR	Lee, 2023
SbFT1, SbFT8, SbFT12 and *SbTIL1*	Vegetative branching	CRISPR	Lee, 2023
SbFT and *SbGA2ox5*	Flowering time	CRISPR	Char *et al*., 2020

Genome editing of oat

Worldwide, the common oat (*Avena sativa*) is cultivated as a cereal. Because of its numerous health advantages, oat has recently gained popularity, which increases its economic value. In particular, oats provide a large source of the dietary fiber (1,3;1,4)- beta-glucan. This dietary fiber has shown promise in lowering dietary intake (Lumaga *et al*., 2012), bacterial and viral infections (Daou and Zhang, 2012), and coronary heart disease (Whitehead *et al*., 2014). Despite these intriguing characteristics, very little is understood about the mechanism and regulation of beta-glucan synthesis. Oats are mostly produced for cattle feed, however there is growing interest in this grain as a potential health food (Kaur and Singh, 2017). The many elements of oat that contribute to its beneficial effects on health are the source of this interest. For instance, the plant *Avena sativa* has powerful antioxidants called avenanthramides. Reactive oxygen species, inflammation, and DNA damage have all been found to be decreased by this potent chemical (Kang *et al*., 2018). Tocopherols (Vitamin E), phenolic acids, vitamin B1, fiber, magnesium, and iron are additional ingredients that contribute to their status as whole foods (Kemppainen *et al*., 2010). Most importantly, the common oat's high content of beta-glucan within the grain greatly contributes to its health advantages. The common oat has three separate ancestral genomes (AACCDD) and is a hexaploid species (2n=6x = 42 chromosomes) (Leggett and Thomas, 1995). The subject of genomics is made harder by this polyploidy. Due to its extremely repetitive genome, the hexaploid oat has been difficult to sequence and characterize. The basis for the area of gene editing is, of course, the availability of functionally defined gene sequences. There hasn't been a lot of research study done on site-

specific nuclease engineering of oats. Donoso (2022) published the first report on this topic. He used the CRISPR/Cas9 technology to investigate the function of Thaumatin-like protein 8 (TLP8). Three carefully crafted constructions were created, each of which was designed to target AsTLP8 A, C, and D, three distinct homeologs. This discovery led to research on TLP8's regulatory function in oat beta-glucan production. The study is noteworthy because it shows that AsTLP8 homeologs in oats can be successfully edited using CRISPR/Cas9. There are other research on oat genome editing application as showed in Table 7.

Table 7. Genome editing application in oat

Genes	Traits studied or Effect	System & strategy	References
AcrIIA4	light-mediated genome and epigenome editing	CRISPR	Bubeck *et al*., 2018
Ac/Ds	Functional genomics studies	CRISPR	Mahmoud *et al*., 2022

Limitations of GE in cereal crops

Any crop that utilizes CRISPR/Cas9 has intrinsic limitations. First, the absence or incomplete assembly of a genome sequence makes it difficult to identify potential targets of interest for editing or assess the off-target activity of gRNAs (Hahn and Nekrasov, 2019). Without knowing the sequence for the target gene, it is also impossible to make the complementary gRNA sequences needed to direct Cas9 nucleases to the target location. Online gRNA design resources can be used to choose candidates with few off targets in order to address off-targeting concerns (Hahn and Nekrasov, 2019). Additionally, these tools can be used to steer clear of potential gRNAs that are ineffective or incompatible with Pol III and have low GC content (Concordet and Haeussler, 2018). The number of viable gRNA candidates is constrained by the requirement for a PAM sequence, which also restricts editing of particular regions of any genome. This is especially restrictive for CRISPR/Cas9 base editing, which currently makes it difficult to target highly specific base edits to many regions of a genome (Mishra *et al*., 2020). For CRISPR/Cas9 base editing, it is necessary to target nucleotides within a base editor catalytic window (typically 4-5 bp, but up to 17 bp) in the vicinity of a PAM sequence. Recently, a nearly PAM-free Cas9 enzyme was created (Walton *et al*., 2020) and used in plants (Ren *et al*., 2021).

When compared to Cas9, results from rice protoplasts show significantly lower editing frequencies (Ren *et al*., 2021). The prohibition of certain nucleotide alterations is another constraint placed on base editing (Mishra *et al*., 2020). Targeted insertions through the HDR route might also be able to get around these base editing restrictions, although HDR is only sometimes seen, which restricts the ability to precisely knock-in gene modifications using donor DNA (Hahn *et al*., 2018). Furthermore, a complex genome structure, including several gene duplications, polyploidy and gene families, is another characteristic of cereal plants. This complex genome structure limits the transformation and regeneration in cereal plants and limit molecular and genetic advancements. *Agrobacterium*-mediated or biolistic bombardment are the two main techniques for stable transformation, however neither technique is appropriate for all crops. Protocols for *Agrobacterium*-mediated transformation and regeneration in cereal plants are difficult to design. Cereal plants are famously challenging to transform; problems can occur with both biolistic transformation and *Agrobacterium*-mediated bombardment techniques for each of these crops. Also necessary for the regeneration of plants from altered tissue are lengthy tissue culture periods (Altpeter *et al*., 2016). Therefore, by enabling the creation of knockout and knock-in lines in plants, multiplex GE is anticipated to be useful to research gene functions (Najera *et al*., 2019; Dong, 2020).

Conclusions

Agriculture and the mass production of crops are the key drivers of the expanding nation's economy. It will be extremely difficult to provide food that is secure, affordable, and nutritious while using sustainable agriculture methods. The availability of cutting-edge technologies to enhance cultivars will be crucial in this regard. GE is a potent technology that is anticipated to be essential in addressing the rising demands for crop output to meet the needs of a rapidly expanding population in the event of climate change. By altering the agricultural genome, genome editing can be utilized to increase crop nutritional value, productivity and create resistance against both biotic and abiotic stresses. Plant genome editing has made considerable use of cutting-edge tools.

Authors' Contributions

The idea originated by NIE and AAA. NIE, AH and AAA wrote the manuscript. NIE developed figures and organized tables. NIE, HSEB, AAR, TAS, MSA, AAM, AH and AAA helped in the literature review. The manuscript was reviewed, revised, and polished by NIE, HSEB, AAR, TAS, MSA, AAM, AH, and AAA. Project administration: HSEB and AAA; Supervision: HSEB and AAA; Funding acquisition: HSEB AAR, TAS, MSA. All authors read and approved the final manuscript.

Ethical approval (for researches involving animals or humans)

Not applicable.

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Conflict of Interests

The authors declare that there are no conflicts of interest related to this article.

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