Malaysian Applied Biology
https://doi.org/10.55230/mabjournal.v54i4.3226

Review Article

Genome Editing for Enhanced Abiotic Stress Tolerance in Selected Cereal (Poaceae) Crops: Current Applications, Tools, and Future Perspectives

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ABSTRACT

Recent progress in genome editing (GEd) technology offers an opportunity to accelerate the breeding of improved crops with enhanced resistance and high tolerance to drought and salinity. In this article, we highlight four programmable site-specific nucleases that are considered prominent GEd technologies: meganucleases, zinc-finger nucleases (ZFNs), transcription activator-like effector nucleases (TALENs), and clustered regularly interspaced short palindromic repeats (CRISPR)/CRISPR-associated (Cas) nucleases. We then focus on the application of CRISPR/Cas9 system and access the transformation methods that have been used to deliver the system into major cereal crops including rice (*Oryza sativa*), maize (*Zea mays*), barley (*Hordeum vulgare*), sorghum (*Sorghum bicolor*), and wheat (*Triticum aestivum*). This review further emphasises the applications of the CRISPR/Cas9 system to impart tolerance to two major abiotic stresses, salinity and drought, in these selected crops. Finally, we summarise bioinformatics tools that are available for cereal genome editing works, including guide RNA (gRNA) design and post-editing analysis tools. This review provides an overview of current progress, identifies research gaps, and offers perspectives for prospective scientists embarking on genome editing in cereals and related crops.

Key words: Abiotic stress, climate change, crop improvement, omics, plant biotechnology, staple food

INTRODUCTION

Poaceae is a grass family that includes the world's most economically important crops: oat (*Avena sativa*), barley (*Hordeum vulgare*), rice (*Oryza* spp.), sorghum (*Sorghum bicolor*), sugarcane (*Saccharum* spp.), wheat (*Triticum aestivum*), and maize (*Zea mays*) (Soreng *et al.,* 2015). Cereals, a group of grain-producing grasses, provide more than 50% of the caloric intake for humans, of which rice, wheat, and maize are the three major cereals cultivated as staples worldwide (Awika, 2011). They are among the earliest crops—cultivated for over 10,000 years—and are planted on over two-thirds of all cultivated lands (Calderini & Slafer, 1998). The grains are produced in multiple locations worldwide and come in various species and types grown to suit different climatic conditions. For instance, wheat and barley thrive in the temperate climates of northern Europe, North America, and parts of Asia. On the other hand, maize and rice thrive in the hotter climates of the tropics (MacNeish & Eubanks, 2000). Rice is native to the humid tropics and is primarily cultivated in Asia. China, India, and Indonesia are the three major rice-producing nations, with an annual output of 443.6 million metric tonnes—more than half of the world's production (OECD/FAO, 2022).

Sustaining a sufficient and nutritious supply of these staples for the growing global population of eight billion people while addressing climate change is genuinely challenging for industrial players, breeders, and crop researchers (Neumann *et al.,* 2010; United Nations, 2022). Climate change affects agriculture worldwide in terms of shifting mean temperatures, extreme weather, unpredictable rain distribution, water availability, and rising sea levels (Gornall *et al.,* 2010). An increased level of the greenhouse effect, which leads to recurrent occurrences of extreme heat and drought, is accountable for agricultural stress. According to Zhao *et al.* (2017), an increase of more than one degree Celsius in ambient temperature is expected to reduce wheat, rice, and maize productivity by 6%, 3.2%, and 7.4%, respectively. A long-lasting answer to such an alarming concern

Article History
Accepted: 11 September 2025
First version online: 15 December 2025
Cite This Article: Tamizi, A.A., Zainal-Abidin, R.A., Md-Yusof, A.A., Mohd-Zim, N.A., Salleh, M.S., Ahmad Azmi, N.S., Zainuddin, Z., Harun, S., Sekeli, R. & Samsulrizal, N.H. 2025. Genome editing for enhanced abiotic stress tolerance in selected cereal (Poaceae) crops: current applications, tools, and future perspectives. Malaysian Applied Biology, 54(4): 1-24. https://doi.org/10.55230/mabjournal.v54i4.3226

would be to develop resilient crops that can tolerate abiotic stressors such as extreme temperatures, water scarcity, and an increase in salinity (Roychoudhury *et al.,* 2013). For those reasons, several technologies, often involving genetic manipulation techniques, have been utilised to improve crops (Lloyd & Kossmann, 2021).

Genetic modification (GM) techniques have been widely employed to develop transgenic plants for trait improvement. The method introduces new genetic materials, including an antibiotic/herbicide resistance gene that acts as a selectable marker trait to assist the selection process during *in vitro* transformation. After selection, the selectable marker gene (SMG) no longer serves any purpose and often poses a metabolic burden to the plant. The presence of the SMG in the cells is also one of the primary reasons genetically modified plants are of public concern and are subjected to government regulations—labelled as genetically modified organisms (GMOs). Working around this constraint, genome editing (GEd) technologies—an alternative strategy to classical GM—have been recently developed and employed to create plants free of foreign DNA materials (Zaidi *et al.,* 2019).

Although the delivery of the Cas gene or protein into cells can be considered an introduction of exogenous elements, the technique does not always result in the incorporation of foreign DNA. During the process, the CRISPR T-DNA (Cas9, gRNA, and selectable marker gene cassettes) is typically introduced into a target organism (such as plant cells) using methods like *Agrobacterium*-mediated transformation or particle bombardment. This T-DNA can be eliminated through segregation during selfing or backcrossing, leaving only the intended genetic modifications and no foreign DNA in the selected progeny or populations (Saha *et al.,* 2025). The final product is then rigorously analysed using molecular techniques, such as standard PCR or whole genome sequencing, to confirm the complete absence of exogenous DNA or proteins. Alternatively, the CRISPR ribonucleoprotein (RNP) complex, which consists of the Cas9 protein and guide RNA, can be directly delivered into cells, entirely bypassing the need for the introduction of foreign DNA (Banakar *et al*., 2019; Sant'Ana *et al*., 2020).

Whether an edited crop is classified as non-GMO ultimately depends on local regulations, particularly if the final product contains no introduced genetic material after editing. Some countries (see *Perspective, Challenges, and Concluding Remarks*) classify organisms edited using Site-Directed Nuclease Type 1 (SDN-1) and SDN-2 techniques, both of which are forms of CRISPR editing, as non-GMO (Saha *et al.,* 2025). These methods make minor, precise modifications to existing genes and produce final products free of foreign genetic material in the genome. In this sense, genome-edited plants are similar to those developed through mutational breeding—a method used for decades to introduce desirable traits in crops (Jung & Till, 2021). However, genome editing stands apart due to its unparalleled precision. Unlike traditional methods, which rely on random mutations, genome editing allows researchers to target and enhance specific traits with a certain degree of accuracy.

Modern GEd technologies were introduced to the scientific world more than a decade ago and have improved various traits in different plant species (Uluisik *et al.,* 2016; Hussain *et al.,* 2018; Farhat *et al.,* 2019; Khalil, 2020; Zaidi *et al.,* 2020; Broothaerts *et al.,* 2021; Wang *et al*. 2024). This effort is significantly propelled by leveraging network biology and bioinformatics, which play a crucial role in selecting target genes and predicting the effects of genome editing (Razalli *et al*., 2025). Despite these advances, the application of genome editing to improve abiotic stress tolerance in major crops of the Poaceae family holds significant potential for further development. This review provides an overview of major GEd technologies for plants, emphasising the CRISPR/Cas9 system. We concentrate on the usage of the GEd in the context of salinity and drought stresses—the two most important abiotic factors that negatively affect agricultural productivity worldwide. In addition, we integrate and compare the available bioinformatics resources that can be utilised for CRISPR-based research in plants from the Poaceae family, which will be helpful for plant breeders. We also discuss the future directions and challenges of utilising GEd technologies in agriculture.

GENOME EDITING TECHNOLOGIES FOR CROPS

Genome editing tools can alter the genomic structure and endogenous genes of a genome in desired and specific regions with great precision. These methods have been effectively used to study gene functions and develop crops with high yields and resilience to biotic and abiotic stresses. Four types of site-directed nucleases (SDNs), constituting a major class of GEd tools, have been used to edit plant genomes: homing endonucleases/meganucleases (EMNs), zinc finger nucleases (ZFNs), transcription activator-like effector nucleases (TALENs), and the CRISPR/Cas system. This study reviews the mechanisms of the four SDNs and their application in plant studies.

Homing endonucleases/meganucleases (EMNs)

According to Abdallah *et al.* (2015), sequence-directed nuclease (SDN) systems involve the binding and cleaving of the target sequence of DNA by a cutting enzyme, leading to gene modifications by cellular DNA repair mechanisms at the target site. The EMNs are one of the four GEd tools under the SDN category. The homing endonucleases/meganucleases were first discovered in the late 1980s and can be found abundantly in prokaryotes, archaea, and unicellular eukaryotes (Abdallah *et al.,* 2015; Iqbal *et al.,* 2020; Dong & Ronald, 2021). This type of nuclease can be programmed through protein engineering and is well-known for its efficiency in cleaving double-strand DNA based on large nucleotide recognition sites (from 14 to 40 base pairs). It was considered the most specific naturally occurring type of restriction enzyme (Stoddard, 2005). There are four well-characterised families of EMNs (the fifth one is not well studied) whose structures, active-site core motifs, and catalytic mechanisms differ extensively. They are LAGLIDADG, GIY-YIG, HNH, and His-Cys, as illustrated in Figure 1 (Stoddard, 2005; Silva *et al.,* 2011; Iqbal *et al.,* 2020).

In 1993 and 1996, a study demonstrated that yeast meganucleases I-SceI generated double-strand breaks (DSBs), increasing the frequency of homologous recombinants at a specific locus by over two orders of magnitude in tobacco cells (Dong & Ronald, 2021). Recently, engineered meganucleases were used in cotton (*Gossypium* spp.) to generate highly effective gene insertions (Iqbal *et al.,* 2020). A few variants of novel meganucleases, exhibiting increased nuclease activity and recognition of diverse sequences, have been produced using specialised mutagenesis and high-throughput screening techniques (Osakabe & Osakabe, 2015). Despite all these benefits (i.e., large recognition sites and high efficiency in cleaving target sequences and gene insertions), this tool still has limitations. In contrast to other SDN systems, meganucleases have the drawback of being more expensive, and sequence-specific enzymes are challenging to synthesise for every potential sequence. Meganucleases are unspecific and may cleave non-target DNA regions, leading to unintended consequences. An initial protein engineering stage

is needed to create a unique meganuclease targeting the desired site, which is technically demanding and hampered by patent challenges.

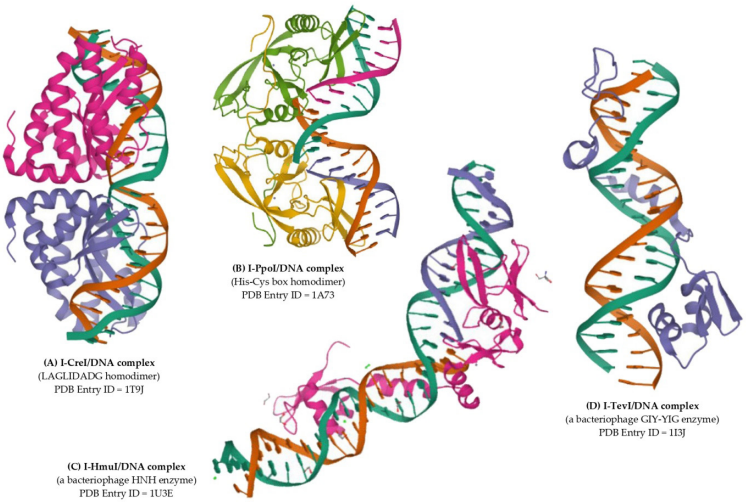

Fig. 1. Three-dimensional (3D) structures of the four types of meganucleases retrieved from Protein Data Bank (PDB, https://www.rcsb.org/). Structures of (A) I-*Cre*I (PDB ID: 1T9J) (Chevalier *et al*., 2004), (B) I-*Ppo*I (PDB ID: 1A73) (Flick *et al*., 1998), (C) I-*Hmu*I (PDB ID: 1U3E) (Shen *et al*., 2004), and (D) I-*Tev*I (PDB ID: 1I3J) (Van Roey *et al*., 2001) in complex with their target DNA helices.

Zinc finger nucleases

Zinc finger nucleases (ZFNs) are hybrid nucleases developed when a DNA-binding domain and a non-specific DNA cleavage domain—derived from endonucleases *Fok*I ZFNs—are combined (Abdallah *et al.,* 2015; Iqbal *et al.,* 2020; Dong & Ronald, 2021). The DNA-binding domain is made up of several zinc finger repeats, which each may recognise a different nucleotide triplet, and a specific nucleotide sequence of 9 to 18 bases can be encoded into the DNA-binding domain by combining different zinc finger repeats (Dong & Ronald, 2021). ZFNs are more adaptive SDNs than the meganucleases; they can be directed to target any genomic region thanks to the properties of the *Fok*I domain, which allows ZFNs to recognise the presence of nearby genomic targets and cut the DNA sequence at specific sites (Dong & Ronald, 2021). Wright *et al.* (2005) transformed ZFNs in tobacco cells through electroporation, including a donor repair template to restore the function of a damaged reporter gene (GUS:NPTII) that had been pre-integrated into the genome. The ZFNs successfully induced indels at the targeted site and homologous recombination (HR) repair of the damaged reporter gene. In addition, the ZFNs have been used successfully in other plants such as Arabidopsis, rice, soy, maize, tobacco, fig, apple, rapeseed, and petunia (Iqbal *et al.,* 2020). However, similar to meganucleases, ZFNs also have their limitations, including complexity and expensive protein domain construction, and it is often challenging when replacing much larger DNA fragments to create gene knockouts (Abdallah *et al.,* 2015; Iqbal *et al.,* 2020).

Transcription activator-like effector nucleases (TALENs)

The editing mechanism of the TALENs also relies on protein-DNA association. It is facilitated by transcription activator-like effectors (TALEs), naturally occurring proteins secreted by bacteria from the genus *Xanthomonas* (Joung & Sander, 2013). TALENs are chimeric proteins comprised of two functional domains: DNA-binding TALE and nuclease domains. The TALEs' motifs are a set of highly conserved ~34 amino acid repeats fused to the *Fok*I endonuclease (Figures 2 and 3B) at the carboxy-terminal. Two hyper-variable residues on the DNA-binding domain recognise a single DNA base and facilitate one-to-one pairing that does not affect the binding specificity of neighbouring TALEs (Gaj *et al.,* 2013). They are known as the repeat variable di-residues (RVDs) domain and are found at positions 12 and 13. Each pair of RVDs binds uniquely to a single nucleotide in the target site's 5' to 3' orientation (Boch *et al.,* 2009). The four most common RVDs identified are HD (Histidine/Aspartic Acid), NI (Asparagine/Isoleucine), NG (Asparagine/Glycine), and NN (Asparagine/Asparagine), with a unique preferential binding affinity towards C, A, T, and G/A, respectively (Mussolino & Cathomen, 2012). HD, NI, and NG are all hydrophilic amino acids that are believed to interact with the phosphate groups of DNA, while NN is thought to stabilise the overall structure of the TAL effector protein. Similar to ZFNs (Figure 3A), the cleaving of a target DNA site will only occur once the *Fok*I endonuclease of the TALENs on both opposite strands dimerises (Figure 3B).

TALENs are preferred over ZFNs for being less cytotoxic and easier to engineer (Malzahn *et al.,* 2017). Initially, the complex construction of the repetitive sequence of the TALE via polymerase chain reaction (PCR) hindered the adoption of TALEN. However, the system has been further improved with the development of multiple assembly methods, mainly through modular Golden Gate cloning (Li *et al.,* 2011). Over the years, TALENs have been fine-tuned to increase their specificity. The identification and characterisation of new non-conventional RVDs have demonstrated an efficient TALEN-mediated editing process and reduced undesired off-site cleavage of a specific target sequence (Zhang *et al.,* 2016; Juillerat *et al.,* 2015; Miller *et al.,* 2015). The application of TALENs as a GEd tool has become widespread in various organisms, including plants of the Poaceae family, to modify endogenous genes for proof-of-concept, trait improvement, and gene functional studies. TALENs have been demonstrated to be functioning in regulating the expression of genes responsible for the response to abiotic and biotic stresses in rice, wheat, maize, and barley (Liang *et al.,* 2014; Wang *et al.,* 2014; Budhagatapalli *et al.,* 2015; Zhang *et al.,* 2016;

Takáč *et al.,* 2021).

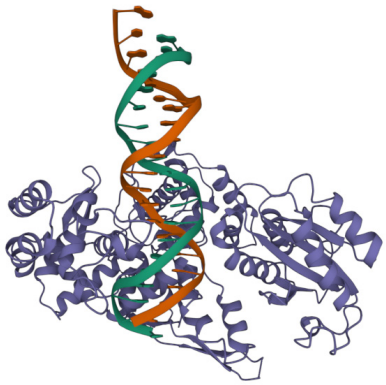

Fig. 2 Three-dimensional structure of the restriction endonuclease *Fok*I (PDB Entry ID:1FOK) (Wah *et al*., 1997) bound to DNA. The endonuclease domain of *Fok*I (purple ribbon) has been used in combination with various DNA-binding domains (not shown), such as the zinc finger (ZF) and transcription activator-like effector (TALE), to form a zinc finger nuclease (ZFN) and a transcription activator-like effector nuclease (TALEN), respectively. The structure was generated by searching the protein name/ID on Protein Data Bank (PDB).

Clustered regularly interspaced short palindromic repeats (CRISPR)

Clustered regularly interspaced short palindromic repeats (CRISPR) and their associated protein (Cas) are small RNA-based prokaryotic innate defence systems that remember previous infections. It works by integrating short genetic sequences—called spacers—derived from invading phages or other foreign genetic materials into the CRISPR locus, a kind of adaptive immunity machinery activated by the host cells (Mojica *et al.,* 2005; Makarova *et al.,* 2006; Barrangou *et al.,* 2007). These spacers are then expressed as small guide CRISPR RNAs (crRNAs), also known as guide RNAs (gRNAs) or single gRNAs (sgRNAs). In turn, Cas proteins employ them to target specific intruding sequences upon infection. According to Hille *et al.* (2018), this defence system is present in approximately 50% of bacteria and 90% of archaea. Surprisingly, more CRISPR systems are being discovered in viruses (Al-Shayeb *et al.,* 2022). The authors reported the extensive presence of diverse and compact CRISPR/Cas systems within phage genomes, discovered through metagenomic analysis. Notably, these systems exhibited intrinsic genome-editing capabilities in both plant and human cells.

The *Streptococcus pyogenes* CRISPR/Cas9 system (CRISPR-*Sp*Cas9) was the first to be created for genome editing, and the term “CRISPR/Cas9” is frequently used to refer to this system. However, the *Sp*Cas9 nuclease is one of many kinds in an intricate CRISPR world. The CRISPR/Cas system is functionally categorised into two principal classes—class 1 and class 2—based on the composition of the effector genes/proteins, and these classes are further divided into different types (Makarova *et al.,* 2015; Moon *et al.,* 2019). The effector module in class 1 CRISPR/Cas systems employs multiple Cas proteins, whereas class 2 systems employ only one Cas protein to recognise and cleave nucleic acids (Makarova *et al.,* 2015; Moon *et al.,* 2019). According to Moon *et al.* (2019), utilisations of the class 1 CRISPR system—including the type I, III, and IV CRISPR systems—for GEd have been limited due to its complexity, restraints, and a lack of knowledge about suitable cloning systems involving a functional vector and the utilisation of a ribonucleoprotein (RNP) complex. With their multi-protein nuclease complexes, class I CRISPR/Cas system—including Type I and Type III—can cut and processively degrade the target DNA or RNA, leading to large deletions or extensive damage (Liu & Doudna, 2020). To date, only one notable study by Wada *et al*. (2023) has demonstrated the use of a type I-D (TiD) CRISPR/Cas system to introduce small indels and long-range DNA deletions in tomato. As mentioned, class 2 CRISPR/Cas system requires only a single effector nuclease—the Cas protein—to function, and routine practice of GEd has been achieved thanks to the advancement of this CRISPR category, which includes the type II, V, and VI CRISPR/Cas systems (Moon *et al.,* 2019). The type II and V systems can be utilised for DNA editing, whereas the type VI system is employed for RNA editing (Makarova *et al.,* 2015; Moon *et al.,* 2019). CRISPR/Cas9 belongs to class 2 and falls under the type II system.

The CRISPR/Cas9

The CRISPR system has been described as an innate immune system of several microorganisms, and Francisco Juan Martínez Mojica was one of the early researchers to shed light on the existence and potential function of CRISPR sequences (Mojica *et al.,* 2005). Building on this foundation, Jennifer Doudna and Emmanuelle Charpentier proved that the CRISPR/Cas9 system derived from *S. pyogene*s could be used to cleave the DNA strand at any desired location (Jinek *et al.,* 2012; Le Rhun *et al.,* 2019). This invention was so significant and versatile that both researchers were awarded the Nobel Prize in Chemistry in 2020 (Westermann *et al.,* 2021). Because the site-specific catalytic action of this gRNA-Cas9 complex is defined by a sequence of only ~20 consecutive nucleotides of gRNA (Dagdas *et al.,* 2017), CRISPR/Cas9 is more accessible and easier to manipulate than its predecessors (the ZFN and TALEN systems) (Figure 3). The CRISPR/Cas9 system has since opened new avenues in GEd in a wide range of living species with promising applications in therapeutics, agriculture, food industries, and bioenergy (Nidhi *et al.,* 2021).

In CRISPR/Cas9 systems, a gRNA forms a complex with Cas9. The base complementation of the gRNA with DNA (Figures 3C and 4) determines the homing specificity of Cas9 in causing a DNA break in the targeted nucleotide region (Bhandawat *et al.,* 2020). In addition, the protospacer adjacent motif (PAM) NGG is required by Cas9 to create the DSB (Figure 3C), which is a slight limitation but also contributes to the specificity of the system (Cong *et al.,* 2013). Once the Cas9-gRNA complex introduces

a DSB, the host cell will execute its innate DNA repair system to fix the splitting, which is error-prone. This will result in alterations such as the introduction of indels and SNPs (Song *et al.,* 2021). Previous works have indicated the successful application of this system for trait improvement in tomato (*S. lycopersicum*), poplar (*Populus trichocarpa*), citrus (*Citrus sinensis*), sorghum (*S. bicolor*), maize (*Z. mays*), and wheat (*T. aestivum*) (Liu *et al.,* 2019; Young *et al.,* 2019; Wang *et al.,* 2019a; Wang *et al.,* 2020; Brandt *et al.,* 2020; Dutt *et al.,* 2020). In the next section, we highlight the GEd work on major food crops (Poaceae) pertaining to salinity and drought tolerance.

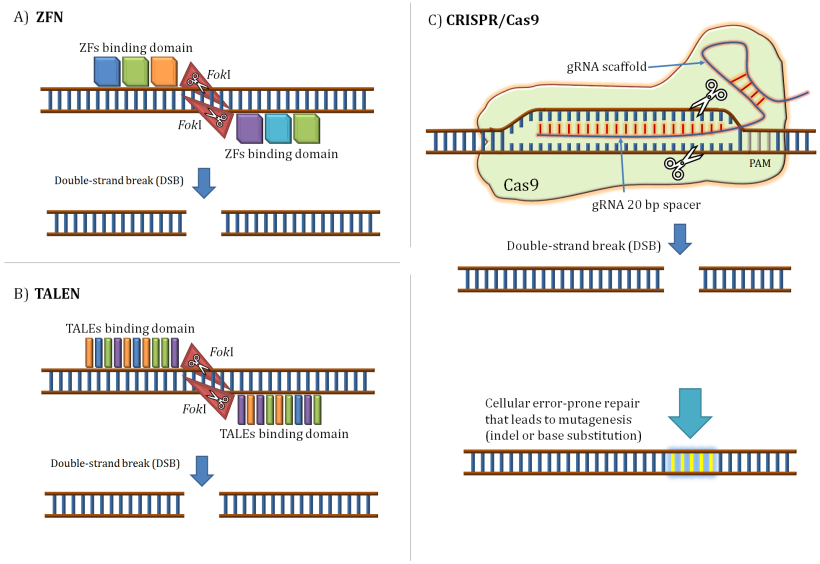

Fig. 3. Schematic representations of the architecture and editing mechanisms of three different GEd nucleases. (A) ZFN, (B) TALEN, and (C) CRISPR/Cas9 introduce DSBs at the targeted sites that will be automatically rejoined via non-homologous end-joining (NHEJ) repair or homology-directed repair (HDR) systems with the presence of a donor template. Indels, or SNPs, are spontaneously generated due to the nucleotide disruption and cellular self-repair mechanisms caused by the NHEJ at the cleaved site. The heterodimerisation of ZFNs and TALENs on the target DNA initiates the DSB, while complementing the gRNA to the target DNA leads Cas9 to cleave the strand.

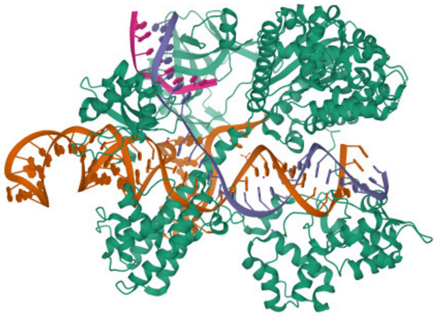

Fig. 4. Three-dimensional conceptual binding of the Cas9-gRNA complex (PDB Entry ID: 5AXW) (Nishimasu *et al*. 2015) to the target DNA double strands.

CRISPR/Cas9 delivery platforms for genome editing in plants

The CRISPR/Cas9 tool surpasses its congeners in terms of efficiency and simplicity; thus, various plant-specific CRISPR/Cas9 delivery systems have been developed for different plant species. Compared to bacterial and mammalian cells, delivering CRISPR/Cas9 components through the rigid plant cell wall is challenging and requires different approaches depending on the type of explants and the species being targeted (Laforest & Nadakuduti, 2022). The most common methods for delivering the CRISPR components into plant cells (especially cereals) are bombardment (or biolistic) transformation, *Agrobacterium*-mediated transformation, and protoplast-based transformation (Table 1). In addition, Michalski *et al.* (2023) demonstrated that wheat cell suspension culture could be transformed with CRISPR/Cas9 constructs using the *Agrobacterium*-based delivery method, providing more options to researchers working with complex hexaploid species. However, these methods mostly rely on conventional regeneration procedures to generate the whole plant, which is time-consuming and often laborious (Hwang *et al.,* 2017; Sandhya *et al.,* 2020; Miller *et al.,* 2021; Laforest & Nadakuduti, 2022; Michalski *et al.,* 2023).

Previous efforts have shown that an *in planta* transformation method successfully delivered the CRISPR system in wheat, enabling the bypassing of tissue culture and regeneration steps (Hamada *et al.*, 2018; Imai *et al.*, 2020). Recently, we have proven that the *Cas9* gene can be transformed into the rice meristem (coleoptile) of sprouting seeds using *Agrobacterium*-mediated *in planta* transformation (Tamizi *et al.*, 2023). These significant advancements have paved the way for the application of the same concept to other members of the Poaceae family. Viswan *et al.* (2022) demonstrated a novel method for delivering Cas9 RNP into *Arabidopsis thaliana* and *Glycine max* (soybean) tissues using a microneedle array, and this approach holds potential applicability to cereal crops as well. In addition, Sandhya *et al.* (2020) discussed two more potential methods, including nanoparticle-mediated and pollen magnetofection-mediated deliveries, which are independent of tissue culture procedure.

Table 1. Delivery methods of CRISPR/Cas9 components in various grain species and one perennial grass species (sugarcane), targeting different genes and regulatory elements. The CRISPR/Cas9 system can be delivered into the cells as gene cassettes/plasmids (DNA form) or Cas9-gRNA ribonucleoprotein (RNP) complexes (protein form), depending on the type of delivery platform used.

Particle bombardment delivery for CRISPR/Cas9 components			
Crop species	CRISPR/Cas9 vector or RNP complex	Target gene or regulatory element	Reference
Hordeum vulgare	pcas9:gRNA	ENGase	Kapusi *et al.* (2017)
Oryza sativa	pYLsgRNA-OsU6a, pYLsgRNA-OsU6b	*OsFAD2-1*	Bahariah *et al.* (2021)
Oryza sativa	pCam1300-CRISPR-B	*crtI*, *ZmPsy*	Dong *et al.* (2020)
Oryza sativa	CRISPR-RNP complex	*OsPDS1*	Banakar *et al.* (2019)
Oryza sativa	pJIT163-2NLSCas9	*OsPDS, OsBADH2*	Shan *et al.* (2013)
Oryza sativa	pOsU3-sgRNA	*OsDEP1*	Shan *et al.* (2014)
Saccharum spp. hybrid	Five plasmids carrying various DNA elements	*ALS*	Oz *et al.* (2021)
Sorghum bicolor	pCUBi1390-Cas9	*SbCAD*	Liu *et al.* (2019)
Triticum aestivum	pE(R4-R3)ZmUbi_OsCas9_ver3, pTAKN-sg-GR7	*TaGASR7*	Hamada *et al.* (2018)
Zea mays	Various plasmids carrying different DNA elements	*ARGOS8* promoter and 5' UTR	Shi *et al.* (2017)
Zea mays	pSB11-Ubi:Cas9	*LIG1*, *Ms26*, *Ms45*, *ALS1*, *ALS2*	Svitashev *et al.* (2015; 2016)

Agrobacterium-mediated delivery of CRISPR/Cas9 components				
Crop species	CRISPR/Cas9 vector	*Agrobacterium* Strain	Target gene	Reference
Hordeum vulgare	pSH91, p6i-d35S-TE9	AGL1	*HvMPK6*	Křenek *et al.* (2021)
Hordeum vulgare	pYLsgRNA-OsU6, pYLCRISPR/Cas9Pubi-H	AGL1	*HvITPK1*	Vlčko & Ohnoutková (2020)
Hordeum vulgare	pICSL11056, pHvL1P3GA, pHvL1P4GA, pAGM8031	AGL1	Undisclosed	Lawrenson & Harwood (2019)
Oryza sativa	pRGEB32	EHA105	Undisclosed	Tamizi *et al.* (2023)
Oryza sativa	pC1300-POsU3::sgRNA1-PUQ::SpCas9n-POsU3::sgRNA2	EHA105	*OsDST*	Santosh Kumar *et al.* (2020)
Oryza sativa	VK005	EHA105	*ISA1*	Shufen *et al.* (2019)
Oryza sativa	YLCRISPR/Cas9Pubi-H, pYLgRNA	-	*OsRR22*	Zhang *et al.* (2019)
Oryza sativa	pC-ERF922, pC-ERF922S1S2, pC-ERF922S1S2S3	EHA105	*OsERF922*	Wang *et al.* (2016)
Sorghum bicolor	pCas9:GFP, pgRNA1, pTF101.1	AGL1	*SbGA2ox5, SbFT*	Do *et al.* (2016), Char *et al.* (2020b)
Sorghum bicolor	pVS1 binary vector derived from pLH7500	Y158	*DsRED2*	Jiang *et al.* (2013)
Triticum aestivum	pBI121	GV3101	*Inox*, *PDS*	Upadhyay *et al.* (2013)
Zea mays	pGW-Cas9 derived from pMCG1005	EHA101	*Argonaute 18*, *Dihydroflavonol-4-reductase*	Char *et al.* (2017)

Protoplast-based delivery of CRISPR/Cas9 components			
Crop species	CRISPR/Ca9 vector or RNP complex	Targeted gene	Reference
Oryza sativa	pHUN411	*OsLTPg29*	Li *et al.* (2021)
Oryza sativa	pRGE3, pRGE6	*OsMPK5*	Xie & Yang (2013)
Oryza sativa	pUC19-OsCas9	*OsSWEET14*, *OsSWEET11*	Jiang *et al.* (2013)
Oryza sativa	pJIT163-2NLSCas9	*OsPDS*, *OsBADH2*	Shan *et al.* (2013)
Triticum aestivum	pJIT163-Ubi	*TaMLO-A1*	Wang *et al.* (2014)
Zea mays	pBUE411	*ZmLTPg11, ZmLTPx2*	Li *et al.* (2021)
Zea mays	RNP complex	*IPK*	Sant'Ana *et al.* (2020)

APPLICATION OF GENOME EDITING TO IMPROVE TOLERANCE AGAINST SALINITY AND DROUGHT IN SELECTED CROPS OF THE POACEAE FAMILY

Several studies have been performed for developing abiotic stress tolerance crops using genome editing approach. In this section, we summarise and discuss previous works on GEd with an emphasis on the CRISPR-based system for the development of abiotic stress tolerance in the major cereal crops: rice, maize, wheat, and barley (Table 2).

Rice

According to Awan *et al.* (2017), over 40,000 rice varieties—including cultivars, landraces, and heirlooms—are planted on all continents (except Antarctica), and almost half of the global population depends on rice as the primary source of staple food (Linares, 2002; Van Andel, 2010; Radin Firdaus *et al.*, 2020). Due to its importance as a primary staple food, the number of studies on rice GEd surpasses those on other cereals (Zainuddin *et al.*, 2021).

To date, 12 potential genes have been used in genome editing works to improve tolerance towards salinity and drought in rice (Table 2). Alam *et al.* (2022) improved salinity tolerance by introducing a single-base deletion in the rice basic helix-loop-helix protein 24 (*OsbHLH024*) gene. The mutation significantly increased the shoot weight, total chlorophyll content, and chlorophyll fluorescence, even when exposed to high salinity. Zhang *et al.* (2019) and Han *et al.* (2022) induced mutations in the response regulator (RR) transcription factor 22 (*OsRR22*) gene and managed to recover rice lines with improved salinity tolerance without taking a toll on other agronomic traits. Wang *et al.* (2019b) utilised CRISPR/Cas9 to introduce double mutations in two RR genes—*OsRR9* and *OsRR10*—and the produced mutant 'Kitaake' rice seedlings displayed a higher salinity tolerance than the wild-type seedlings. Even though the mutants displayed a standard growth rate, there was a slight reduction in the number of spikelets per panicle compared to wild-type plants. Additionally, rice with knocked-out drought and salinity tolerance (*OsDST*) and microRNA 535 (*OsmiR535*) genes also exhibited significant tolerance to salt stress (Santosh Kumar *et al.*, 2020; Yue *et al.*, 2020).

Tolerance to salinity and drought is a polygenic trait governed by multiple genes. Responses to these stresses can have overlapping gene regulation pathways (Shelake *et al.*, 2022). Lan *et al.* (2019) produced SBP-like 10 (*OsSPL10)* knockout mutants in 'Huanghuazhan' and 'Zhonghua 11' rice, leading to significant salt tolerance and a lack of trichomes in leaves and glumes. Santosh Kumar *et al.* (2020) reported that the 'MTU 1010' mega rice with mutated *OsDST* exhibited water retention ability under drought stress, in addition to its previously reported increased salinity tolerance. Kim *et al.* (2023) also successfully generated several 'Dongjin-byeo' *japonica* rice lines with an improved tolerance towards drought and salinity stress. Yue *et al.* (2020) confirmed that the *OsmiR535* mutant 'Nipponbare' seedlings have an increased survival rate to PEG-induced drought. It was also reported that CRISPR/Cas9 editing of the pyrabactin resistance-like 9 (*OsPYL9*) gene could enhance drought tolerance and yield in 'IR-96' rice (Usman *et al.,* 2020). Meanwhile, the mutagenesis of enhanced response to the ABA 1 (*OsERA1*) gene by CRISPR/Cas9 resulted in high drought stress tolerance in 'Nipponbare' rice (Ogata *et al.,* 2020). Liao *et al.* (2019) performed CRISPR/Cas9 editing on two SEMI-ROLLED LEAF genes (*SRL1* and *SRL2*) in 'GXU' rice lines and managed to confer drought tolerance and a higher grain filling percentage. However, the mutant rice exhibited rolled leaf morphology, which could be reduced through hybridisation. The gene encoding the Small Ubiquitin-like Modifier (SUMO) E2-conjugating enzyme, known as *OsSCE1*, stands as a promising candidate for CRISPR/Cas9 knockout in both *japonica* and *indica* rice to confer drought tolerance. This potential gene has been highlighted in studies by Nurdiani *et al.* (2018) and Md Yusof *et al.* (2023).

Maize

Maize (*Zea mays* L.) is one of the most widely grown crops for food, animal feed, and biofuel (Ranum *et al.,* 2014; Choudhary *et al.,* 2020). Its output is significantly influenced by a range of meteorological and soil factors, including salinity, drought, heat, and chilling stresses (Derby *et al.,* 2005; Esim & Atici, 2016). Through BAC sequencing, concerted genome mapping, and genome-wide association studies, plant scientists are able to identify genes and genomic regions connected to desirable traits in maize. They may utilise this information through genome editing to increase crop quality, production, and resistance to biotic and abiotic challenges. Liu *et al.* (2020) recovered three independent lines of *ahb2*-CRISPR knockout maize, indicating significant viability compared to wild-type plants after a drought test. They concluded that the mutation of this non-symbiotic haemoglobin (*Hb*) gene imparted a faster stomatal closure to minimise water loss when the plants were dehydrated. By employing Cas9 and a dual sgRNAs system, Shi *et al.* (2017) replaced the native promoter of the AUXIN-REGULATED GENE INVOLVED IN ORGAN SIZE (*ZmARGOS8*) with the constitutive promoter of G0/G1 switch 2 (*GOS2*) gene, producing new edited maize lines. This swapping of the native *ZmARGOS8* promoter led to an increased expression of *ZmARGOS8* in maize. Field tests indicated that these new maize variants exhibited enhanced grain yield under drought stress. In addition, field trials have demonstrated that silencing the 1-aminocyclopropane-1-carboxylic acid synthase 6 gene (*ACS6*) increased grain production in transgenic maize plants under drought stress (Habben *et al.,* 2014). Thus, the *ACS6* gene is a good candidate for mutagenesis using the CRISPR platform.

Wheat

The production of wheat (*Triticum aestivum* L.), ranking as the world's third largest crop after maize and rice (OECD/FAO 2022), is majorly affected by abiotic stresses, including high temperatures, drought, and salinity (Abhinandan *et al.,* 2018). To address the challenges caused by these stresses, researchers have established the GEd approach for wheat as well. Kim *et al.* (2018) successfully demonstrated the CRISPR/Cas9 GEd system in wheat protoplasts targeting two abiotic stress-responsive transcription factor (TF) genes: wheat ethylene-responsive factor 3 (*TaERF3*) and wheat dehydration responsive element binding protein 2 (*TaDREB2*). Both are involved in the positive regulation of drought stress. Using the CRISPR/Cas9 system, Zheng *et al.* (2021) introduced a mutation to the histone acetyltransferase gene (*TaHAG1*) in hexaploid wheat and determined the gene as a positive regulator of salt tolerance in wheat. However, these knockout studies did not indicate improved tolerance to abiotic stresses. On the contrary, Kim *et al.* (2018) stated that the overexpression of *DREB* members could increase drought resistance in wheat, barley, Arabidopsis, and soybean. Inconsistently, Chowdhury *et al.* (2022) pointed out that the mutant wheat lines harbouring the edited wheat TF genes (*TaERF3* and *TaDREB2*) had enhanced drought tolerance.

A recent review on GEd in crops only included the study of genome-edited wheat sensitive to salinity and drought stresses, thus demonstrating the need for GEd studies on wheat with improved tolerance towards abiotic stresses (Shelake *et al.,* 2022). So far, one study has successfully imparted drought tolerance in wheat. Abdallah *et al.* (2022) employed a multiplex-CRISPR/Cas9 editing system by targeting five 3'(2'),5'-bisphosphate nucleotidase (*TaSal1*) homologous genes to create mutants in wheat. They screened the mutants using polyethylene glycol (PEG) and demonstrated that they grew better than wild-type plants under induced drought conditions.

Barley

Barley (*Hordeum vulgare* L.) is one of the oldest cereal crops known to be cultivated and is currently regarded as an important cereal crop after rice, maize, and wheat. It is widely used for animal feed and has some purposes in the human diet and the brewing industry (Harwood, 2019). Barley, a diploid organism with seven chromosome pairs (2n = 14), is also widely used as a genomic model of the Triticeae tribe with good adaptation to various abiotic stresses (Saisho & Takeda, 2011; Arshadi *et al.,* 2018). The capacity of barley to withstand multiple abiotic stresses implies strong DNA-binding specificity of TFs in the transcriptional regulation of stress-responsive genes (Gürel *et al.,* 2016). Through comparative genomics and gene overexpression, the same study identified several stress tolerance-related TF proteins and genes encoding Late Embryogenesis Abundant (LEA) proteins, transporters, osmolytes, and antioxidant enzymes in barley. Al Abdallat *et al.* (2014) reported that the overexpression of the stress-responsive NAC1 gene (*HvSNAC1*) in barley leads to drought tolerance. In Arabidopsis, the overexpression of the barley ethylene response factor (*HvERF*) and root abundant factor (*HvRAF*) confers pathogenic resistance and salt tolerance, respectively (Jung *et al.,* 2007). Lawrenson & Harwood (2019) and Kapusi *et al.* (2017) reported successful targeted mutagenesis in barley using the CRISPR/Cas9 system through *Agrobacterium*-mediated and biolistic transformations; however, reports on edited barley with improved tolerance towards abiotic stresses remain limited.

Gasparis *et al.* (2019) employed the CRISPR/Cas9 system to target the cytokinin oxidase/dehydrogenase 1 gene (*HvCKX1*) in 'Golden Promise' barley and reported several deletion mutant lines. Ten-day-old mutant seedlings had roots with increased length, higher density, and more lateral hairs. The authors did not report or discuss the tolerance level of the mutant barley lines towards any abiotic stress. However, the relationship between improved root architecture and drought tolerance is well-known (Comas *et al.,* 2013). Later, Vlčko & Ohnoutková (2020) produced mutant barley lines with a mutated inositol trisphosphate 5/6 kinase 1 (*HvITPK1*) gene using the programmable nuclease Cas9. They reported that an insertion mutant (*itpk1-2*) was more tolerant than the wild-type to high salinity stress (200 mM of sodium chloride) during germination. However, the mutant line had poor growth compared to wild-type seedlings under zero and low salt concentrations.

Sorghum

Sorghum (*Sorghum* spp.) is a group of crops with considerable economic importance due to its usage as a primary food source and a starting ingredient in food production. *Sorghum bicolor*, native to Africa, is a species widely grown for food (in the form of grain or sorghum syrup), alcoholic drinks, animal feed, and biofuels (Clottey *et al.,* 2014). Igartua *et al.* (1994) state that sorghum has wide-ranging adaptability and robust stress resistance. Regarding application, GEd work on sorghum lagged behind other cereals, hampered by low and inconsistent transformation efficiency (Char *et al.,* 2020a). Nevertheless, several studies of CRISPR/Cas9 targeting centromere-specific histone 3 (*SbCENH3*), the *k1C* family, FLOWERING LOCUS T (*SbFT*), and gibberellin 2-beta-dioxygenase 5 (*SbGA2ox5*) genes have been reported in sorghum (Che *et al.,* 2018; Li *et al.,* 2018; Char *et al.,* 2020b). More recently, Brant *et al.* (2021) described successful CRISPR/Cas9-mediated targeted mutagenesis of the liguleless-1 (*LG1*) gene, which led to phenotypic changes in the mutants, such as varying leaf inclination angles. Even though sorghum is ranked as the fifth most important cereal crop and is considered drought-resistant (Abdel-Ghany *et al.,* 2020), this high-energy grain crop receives less attention in the GEd study on abiotic stresses.

Other related crops

Several GEd studies are also conducted on other crops from the Poaceae family, but they are unrelated to abiotic stress tolerance traits. Oat (*A. sativa*) is a minor cereal crop among the economically essential grains (Ruja *et al.,* 2021). Currently, there is no reported work on GEd to improve abiotic stress tolerance in this highly nutritious cereal. The first and only work on oat GEd was done by Donoso (2021), who successfully produced knockout mutants of *AsTLP8,* a gene related to the beta-glucan synthesis pathway. Millets are a group of small-grained grasses primarily consumed in Asia and Africa that are gaining attention due to their high nutritional content (Saleh *et al.,* 2013). Liang *et al.* (2021) established highly efficient GEd systems—CRISPR/Cas9 and base editors—for foxtail millet (*Setaria italica*), targeting single and multiple genes. Given the success in producing mutations in different genes, this work will open new opportunities to study abiotic stress tolerance traits in millets in the future. Finally, Hussin *et al.* (2022) provide the most recent overview of CRISPR/Cas9 GEd works on sugarcane (*Saccharum* spp.), an economically important non-cereal crop of the grass Poaceae family. Nevertheless, none of the studies are related to abiotic stress tolerance.

THE DEVELOPMENT OF BIOINFORMATICS TOOLS AND DATABASES FOR THE IMPLEMENTATION OF CRISPR-BASED EXPERIMENTS IN CEREAL CROPS (POACEAE)

The increasing adoption of CRISPR/Cas in life sciences has brought about the parallel expansion of a new bioinformatics tool ecosystem. This state-of-the-art bioinformatics tools, databases, and algorithms will boost CRISPR-based experiments for crop improvement. According to Sledzinski *et al.* (2020), three major computational tools associated with CRISPR/Cas have proven convenient for researchers. The first group includes programmes helping researchers select target sites on their genes of choice and design gRNAs. The second group comprises tools developed to predict the outcomes of CRISPR/Cas edits based on repair biases before the GEd work. Finally, the last group of CRISPR/Cas-associated tools analyses post-editing outcomes, such as off-targets, indels, and homologous recombination events.

Although various computational tools have been developed for CRISPR-based experiments on monocots, a comprehensive summary of their functions and features must still be provided, and information on the relevant bioinformatics tool for the Poaceae family crop species needs to be highlighted. In addition, biologists need help selecting suitable tools and appropriate parameters for their analyses. Therefore, providing a detailed review of the existing bioinformatics tools, databases, and algorithms for CRISPR-based experiments is essential. Here, we summarise the features of several computational tools, covering major web-based prediction tools and algorithms dedicated to designing gRNAs, predicting the outcomes of CRISPR/Cas edits, and analysing post-genome editing in cereals. In addition, we reviewed some essential CRISPR/Cas databases that ought to be useful for researchers involved in plant genome editing. Here, we include 13 computational tools available for predicting gRNA,

two for predicting CRISPR/Cas editing, and three for analysing CRISPR post-editing. Additionally, three CRISPR/Cas web-based databases for plants are available for users.

Table 2. Summary of the different genes targeted for editing to improve tolerance towards salinity and drought stresses in selected species of the Poaceae family (rice, maize, wheat, and barley).

Plant Species	Edited Gene	Editing System	Type of stress	Reference
Oryza sativa	*OsbHLH024*	CRISPR/Cas9	Salinity	Alam *et al.* (2022)
	OsRR22	CRISPR/Cas9	Salinity	Zhang *et al.* (2019) and Han *et al.* (2022)
	OsRR9, OsRR10	CRISPR/Cas9	Salinity	Wang *et al.* (2019b)
	OsSPL10	CRISPR/Cas9	Salinity/Reduced trichomes	Lan *et al.* (2019)
	OsPUB7	CRISPR/Cas9	Salinity/Drought	Kim *et al.* (2023)
	OsDST	CRISPR/Cas9	Salinity/Drought	Santosh Kumar *et al.* (2020)
	OsmiR535	CRISPR/Cas9	Salinity/Drought	Yue *et al.* (2020)
	OsPYL9	CRISPR/Cas9	Drought	Usman *et al.* (2020)
	OsERA1	CRISPR/Cas9	Drought	Ogata *et al.* (2020)
	SRL1 and *SRL2*	CRISPR/Cas9	Drought	Liao *et al.* (2019)
Zea mays	*abh2*	CRISPR/Cas9	Drought	Liu *et al.* (2020)
	ZmARGOS8	CRISPR/Cas9	Drought	Shi *et al.* (2017)
Triticum aestivum	Five *TaSal1* homologous genes	CRISPR/Cas9	Drought	Abdallah *et al.* (2022)
Hordeum vulgare	*HvITPK1*	CRISPR/Cas	Salinity	Vlčko & Ohnoutková (2020)
	HvCKX1	CRISPR/Cas	Drought (possible)	Gasparis *et al.* (2019)

Guide RNA prediction tools

Designing the gRNA and predicting the on-off target effects are the most critical steps in executing successful gene editing and this process can be challenging, especially when aiming for high specificity and efficiency. Multiple factors contribute to the intricacy of gRNA design, encompassing elements such as target specificity, sequence requirements, secondary structure, delivery and expression considerations, among others. These challenges have led to the development of numerous prediction tools, models, and algorithms, as summarised in Table 3.

CRISPR-P

CRISPR-P (http://crispr.hzau.edu.cn/CRISPR2) is a web-based tool to design a gRNA with minimal off-target potential. It supports gRNA design for 49 well-assembled plant genomes (Lei *et al.,* 2014; Liu *et al.,* 2017). CRISPR-P uses a modified scoring system to rate the efficiency of on-off targeting gRNAs. The parameters for designing a gRNA include a guide sequence (or spacer) with a length in the range of 15–22 nucleotides, a PAM sequence, and small nucleolar RNA promoters. The results are displayed in graphical and tabular formats, and they comprise GC content, microhomology sequence flanking, restriction endonuclease sites, and the secondary structure of the gRNA. Users can upload custom sequences using the "Design" feature if their plant genomes are unavailable.

CRISPRdirect

CRISPRdirect (http://crispr.dbcls.jp) is a web-based tool that allows the user to design and select the gRNA on a genome-wide scale (Naito *et al.,* 2015). Users can input the sequence of interest, choose the PAM requirement (i.e., NGG, NRG, NNGRRT, NG), and select the genome of interest. For example, the Poaceae family genomes on CRISPRdirect include those of maize and rice. The results are displayed in a tabular format with essential information, such as the position of the gRNA, the target sequence, and the number of target sites. Results can be exported as tab-delimited text or in JSON format.

CRISPR-PLANT

The gRNA of rice, maize, and sorghum can be designed using CRISPR-PLANT. The CRISPR-PLANT (http://omap.org/crispr/CRISPRsearch.html) offers a platform for the user to design and build specific gRNAs for CRISPR/Cas9-mediated GEd in model plants (Xie *et al.,* 2014). CRISPR-PLANT uses two PAM motifs (5'-NGG-3' or 5'-NAG-3') to design the gRNA. For off-target assessment, this tool extracts the 20-nucleotide sequences. In addition, CRISPR-PLANT calculates the specificity of all gRNA spacer sequences based on mismatched numbers and positions in their alignments with other spacer sequences. Users can select the species genome and chromosome number from the drop-down list and provide the range or genomic location. The result is displayed in tabular format. It includes information such as the gRNA sequence, minimal mismatch number calculated from all alignments against PAMs, spacers, and PAM sequences, and the location of gRNA target sites in exons, introns, or intergenic regions. Users can also specify the gene identifier if they have a target gene to design.

E-CRISPR

E-CRISPR (http://www.e-crisp.org/E-CRISP/index.html) allows the user to design and evaluate CRISPR ranging from single exons to entire genomes, using fast and accurate algorithms (Heigwer *et al.,* 2014). It also examines the target specificity of the putative designs and assesses their genomic contexts, including exons, transcripts, and CpG islands. E-CRISPR uses a fast indexing approach to ensure rapid annotation of putative gRNA target sites. This algorithm created genome-scale libraries for several organisms within a few hours. Although E-CRISP is available for model organisms, it also helps design gRNAs for

rice, maize, and wheat. This web-based tool provides experiment-oriented design parameters for users: they can input a FASTA sequence or a gene symbol; they can choose relaxed, medium, or strict PAM parameters; they can select single or multiple designs from the drop-down list. The results display potential and successful designs that comprise the input name, the gRNA target sequence and specificity, the annotation and efficiency score, the match string, and several hits per design target. Users can then download the results into a tabular Excel format.

CCTop

CRISPR/Cas9 Target Online Predictor (CCTop, http://crispr.cos.uni-heidelberg.de) is an online tool with a simple user interface to customise the input parameters (Stemmer *et al*., 2015). Through Bowtie (Langmead *et al*., 2009), the tool browses and aligns candidate sgRNAs based on the user's query sequence—gene or exon. It identifies the PAM sites and the complementation of the gRNA spacer sequences. Fewer than six mismatches are allowed in the gRNA spacer to allow successful DSB. In addition, this server allows the user to choose up to 17 PAM motifs corresponding to the CRISPR/Cas system being used, making it one of the most flexible tools available. CCTop analyses off-target sites, assigns a score for each candidate sgRNA—based on the GC content and exon position in the genome reference—and displays an entire output of the candidate sgRNA sites (Figure 5). Many reference genomes are available in the drop-down menu, including different species of animals, bacteria, fungi, plants, protists, and virus. For the Poaceae family, the users can find the genomes of sorghum (*S. bicolor*), foxtail millet (*Setaria italica*), green foxtail (*S. viridis*), barley (*H. vulgare*), rice (*O. sativa* ssp. *indica* and ssp. *japonica*), wheat (*T. aestivum*), and maize (*Z. mays*). Stemmer *et al.* (2015) confirmed through experiments that CCTop could provide reliable sgRNAs for the target gene's knockout, NHEJ, and HDR. Thus, CCTop provides the researchers with a tool for quickly and efficiently identifying high-quality target sites.

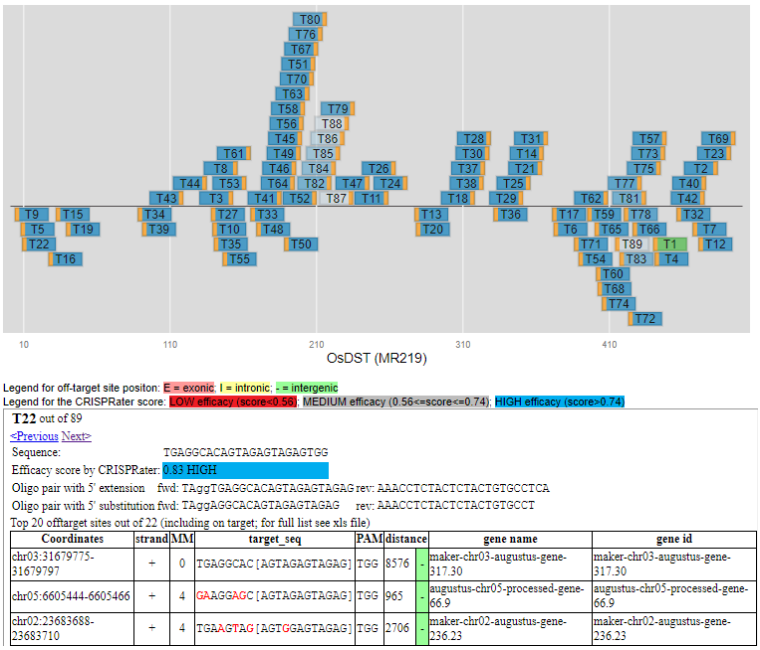

Fig. 5. CCTop displays an output of the candidate editing sites and sgRNAs based on the submitted sequence query. The sgRNAs are scored as LOW, MEDIUM, and HIGH, and the possible off-targets are listed in tables. Image retrieved from CCTop (http://crispr.cos.uni-heidelberg.de).

CGAT

The CRISPR Genome Analysis Tool (CGAT, http://cbc.gdcb.iastate.edu/cgat) allows users to identify potential target sites for CRISPR gene editing in plants (Brazelton Jr *et al.,* 2015). This web-based tool contains several features within a single pipeline, as it enables the user to search by gene name and computes off-targets and ranks the identified targets—the gRNA. To date, CGAT offers gRNA design for maize and rice. Users can select their gene from the database or paste the DNA sequence. If the user chooses to paste the input sequence, they can select the target length: 21 or 23 bp. In addition, the user can set the maximum allowed for nucleotide repeat and the minimum and maximum GC content. The result is displayed in table format and comprises the candidate target sequence, position, unique sequence, and gene identifier.

Breaking-Cas

Breaking-Cas (https://bioinfogp.cnb.csic.es/tools/breakingcas) is a web-based application that allows users to design gRNAs for CRISPR-based experiments and comprises multiple reference genomes available in the ENSEMBL database (Oliveros *et al.,* 2016). Hence, Breaking-Cas is suitable for creating gRNAs for barley, millet, rice, maize, sorghum, and wheat. Compared to other gRNA-designing tools, Breaking-Cas's unique features offer the code for the user to install and set up the programme on their local server. Using Breaking-Cas, the user incorporates several parameters, including mismatch allowance, PAM types and positions, nuclease settings (i.e., Cas nuclease, Cpf1), and oligo length. The results are tabular and can be downloaded in text-delimited format.

Benchling

Benchling (https://www.benchling.com/crispr) is an online tool primarily designed for pharmaceutical and biotech businesses. It assists in the construction of sgRNAs in many organisms, including plants (Pellegrini, 2016; Uniyal *et al.,* 2019; Sladen *et al.,*

2021). The Benchling CRISPR sgRNA design tool allows users to select the preferred reference genome and define the locus of the gene (i.e., chromosomal coordinates). It automatically annotates the coding sequence based on the selected genome. At present, users can choose from 248 reference genomes—from viruses to animals—including the genomes of five cereals: barley (*H. vulgare*), rice (*O. sativa* ssp. *japonica*), sorghum (*S. bicolor*), wheat (*T. aestivum*), and maize (*Z. mays*). Target location, specificity, and efficacy are factors that the server calculates when generating CRISPR sgRNAs. The listing of sgRNAs encompassing on-target and off-target scores (1–100) can be promptly displayed within seconds, and different sgRNA sequences are displayed adjacent to the target gene (Figure 6). The higher the score, the better the sgRNA is. Thanks to its features (i.e., remarkable benchwork interface and visualisation, numerous available reference genomes in the drop-down menu, and reliable support service), Benchling is one of the top CRISPR sgRNA design tools for academic and industry research labs worldwide.

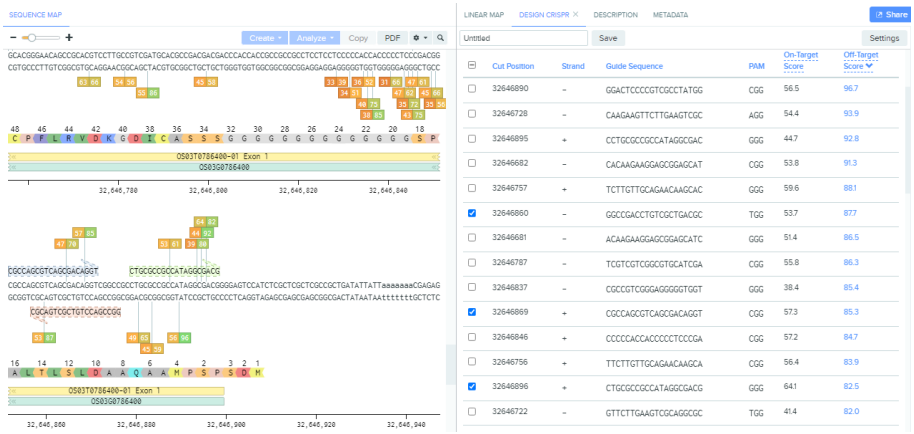

Fig. 6. Benchling CRISPR sgRNA design tool displays outputs on sgRNAs. The sgRNAs are ranked according to user preferences (right) and can be selected to be displayed on the target gene/exon (left). Image retrieved from Benchling (https://www.benchling.com/crispr).

CRISPR-GE

CRISPR-GE (http://skl.scau.edu.cn) is an integrated web-based toolkit for CRISPR-based experiments that houses multiple tools for all steps in the GEd experiment. It contains sub-tools to design sgRNAs for different CRISPR systems (targetDesign, BEtarget, and MMEJ-KO). It can also predict the off-target sites (offTarget), design the primers for the construction of the gRNA expression cassettes (primerDesign), determine mutated sequences from sequencing data (DSDecode), and download genomic sequences of target regions (SeqDownload) (Xie *et al.,* 2017).

In “targetDesign” and “offTarget”, the user can define the PAM type (i.e., NGG, TTN, TTTN) and select the genome of interest. Then, the user can paste the target gene sequence and click the submit button. All the results are displayed in table format, and the user can select the potential gRNA based on the off-score. In “primerDesign”, the user can choose two features that include primerDesign-V and primerDesign-A. PrimerDesign-V is used to design primers for building the sgRNA expression cassettes. In contrast, primerDesign-A is used to develop specific PCR primers to amplify target site-containing genomic fragments.

CRISPR-Local

CRISPR-local (http://crispr.hzau.edu.cn/CRISPR-Local) was developed to design gRNAs for non-reference plant genomes, but it also includes some Poaceae genomes, such as rice, maize, millet, and wheat. Seventy-one plant genomes have been applied in CRISPR-Local to design gRNAs and support Cas9, Cpf1, and custom PAM motifs (Sun *et al.,* 2019). This web-based prediction tool has been developed to design high-throughput gRNAs with high efficiency and deal with the genetic variations among individuals of the same species. Using CRISPR-Local, users can design gRNAs by integrating data from whole-genome and transcriptome sequencings or known variants from specific mutants or lines.

They can also design a gRNA that simultaneously targets multiple genes. Users can select the target genome from the drop-down list by choosing the “DB-Search” feature. They can then select the Cas protein, such as Cas9 or Cpf1 and insert multiple target gene lists in the search box. The results are displayed in tabular format and comprise information such as gene ID, gene position, gRNA sequence for gRNA and off-target genes, score, off-target gene ID, and mismatches.

WheatCRISPR

WheatCRISPR (http://crispr.bioinfo.nrc.ca/WheatCrispr) is a web-based prediction tool allowing users to browse, select, and design an effective gRNA for a specific target gene and predict the potential off-target sites (Cram *et al.,* 2019). This prediction tool applies the Doench algorithm to determine the on-target specificity and potential off-targets for each gRNA targeting specific sites within the wheat genome. This web-based prediction tool features a simple graphical interface (Figure 7) that allows users to search for target gene names, paste sequences, and select an on-target set, coding sequence, or promoter. The results are displayed in three formats: gRNA table, gRNA plot, and gene plot. In addition, users can export the results in CSV or Excel format.

PnB Designer

PnB Designer (https://fgcz-shiny.uzh.ch/PnBDesigner) is written in R and developed with the Shiny Package. It is the first web-based application allowing users to generate gRNAs for CRISPR and base editors (PE, BE), as well as adenine-deaminase base editors (ABEs) (Siegner *et al.,* 2021). In addition, it can design single and multiplex gRNAs to target different genes within a reference genome. Although the PnB designer is developed to design gRNAs in model organisms, such as humans, zebrafish,

and yeast, it also offers candidate gRNAs for rice. The web-based tool is quite simple, with user-friendly features. For instance, the user can choose an editing option (prime or base editing) and a target genome. They can also select the genomes of interest in the Genome panel and input the target sequence by specifying "None of the above" in the Genome panel and Sequence input. Then, the user can select to design gRNAs for PE or BE and for single or multiple edits (multiplex). Results are displayed in a table format with information, such as possible pegRNAs, and the edited base is visualised with bold red text.

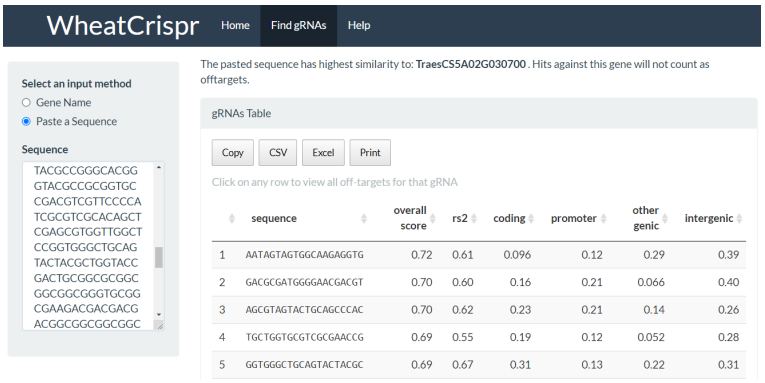

Fig. 7. The WheatCRISPR interface displays the output of candidate sgRNAs generated from sequence input. The sgRNAs are ranked according to the overall score calculated by the tool, and the result can be downloaded in CSV or Excel format. Image retrieved from WheatCRISPR (http://crispr.bioinfo.nrc.ca/WheatCrispr).

CRISPR-Cereal

CRISPR-Cereal (http://crispr.hzau.edu.cn/CRISPR-Cereal) is a gRNA design tool that integrates the information on putative targets of single nucleotide polymorphism (SNP), gene expression profile, and regulome information for maize, rice, and wheat (He *et al.,* 2021). The tool integrates multiple pieces of information to support precise and highly efficient gene editing for rice, wheat, and maize. This web-based tool searches for off-targets genome-wide with a maximally allowed mismatch of up to 5 base pairs. Although a previous gRNA-designing tool (CRISPR-Local) has been developed for wheat, it is unsuitable for grains with a genome size of 16 Gb. Hence, CRISPR-Cereal was developed to encounter this issue. This web-based tool takes less than 49 seconds to scan for off-targets with five mismatches in wheat. Furthermore, the off-target analysis provides valuable information on the off-target sites located on the homologous genes from wheat's A, B, or D sub-genomes. This web-based tool provides a user-friendly graphical user interface (GUI) where the user chooses the "Submit" feature and selects the PAM motifs, such as NGG and TTTN. Users can choose the maximum mismatch and target genome using the drop-down list. Nevertheless, only four target genomes are available: *T. aestivum* (IWGSCv1.1), *O. sativa* ssp. *indica* (MH63), *O. sativa* ssp. *japonica* (IRGSP-1.0), and *Z. mays* (AGPv4). The user can paste custom sequences in the "Sequence" box. This prediction tool provides an output that contains information on the off-targets, such as GC content, position, proximal genes, the position of a gene structure element, and the efficiency score of on-targets and off-targets.

CRISPR/Cas edit prediction tools

The second class of bioinformatics tools includes those providing possible outcomes of CRISPR/Cas edits. Their utility is straightforward compared to the first class of CRISPR tools. Several web tools can predict the editing activity of Cas9 based on sgRNA sequences through machine learning; however, only a few have relevant and proven applications in cereals.

FORECasT

The Favoured Outcomes of Repair Events at Cas9 Targets (FORECasT, https://partslab.sanger.ac.uk/FORECasT) is one of the few available tools that predicts the possible mutational events resulting from DSBs induced by the Cas9-gRNA nuclease (Allen *et al.,* 2019). It computes a forecasted profile of resulting mutations and a percentage of preferred outcomes in the targeted sequence provided by the user. FORECasT utilises a multinomial logistic regression model to predict the probability of the edit outcomes that can have multiple classes (Allen *et al.,* 2019; Molla & Yang, 2019). Allen *et al.* (2019) originally developed this computational tool based on human cell lines, and Molla *et al.* (2022) successfully implemented it in predicting the insertion type in rice.

SPROUT

CRISPR Repair Outcome (SPROUT, https://zou-group.github.io/SPROUT) is another machine-learning system that predicts the repair outcome from a CRISPR/CAS9 knockdown (Leenay *et al.,* 2019). The tool comes with the source code (script) for optimised operation. Users may also utilise the simpler version accessible through their website. In the lighter version, SPROUT accepts the sgRNA sequence (20 bp) followed by the PAM sequence as an input. When running the script (which requires Python 2.7.X or later), the user may choose a second mode that enables genomic factors—in addition to the sgRNA sequence—to be factored in before computing the predicted summary statistics. The repair outcomes that SPROUT predicts for a submitted sgRNA sequence include the fraction of total reads with insertions, insertion/deletion ratios, insertion/deletion lengths, diversity, possible inserted bases, and edit efficiency. First developed based on data from primary human T-cells, FORECasT has also been proven to be applicable in rice (Leenay *et al.,* 2019; Molla *et al.,* 2022).

Table 3. Summary of thirteen web-based tools available for sgRNA design for plants of the Poaceae family. The tools are ordered according to the year of publication.

Tools/Algorithms and URL	Descriptions	Benefits and Limitations	Last Update	References
CRISPR-P http://crispr.hzau.edu.cn/CRISPR2	A web-based tool to design gRNAs of CRISPR-system in plants with minimal off-target potentials. It provides restriction enzyme cutting sites for convenience.	Suitable for general gRNA design for 49 plant genomes. It offers graphical and tabular outputs with detailed information (e.g., GC content, restriction sites, secondary structure). Although it provides a modified scoring system to evaluate on - and off-target, it may not be as advanced as some newer tools.	October 2016	Lei *et al.* (2014); Liu *et al.* (2017)
CRISPRdirect http://crispr.dbcls.jp	A web-based tool allowing users to design and select the candidate gRNA on a genome-wide scale.	Suitable for general gRNA design in major crops. It displays the results in a tabular format with detailed information, making it easier for users to download them.	November 2020	Naito *et al.* (2015)
CRISPR-PLANT http://omap.org/crispr/CRISPRsearch.html	A web-based tool to perform genome-wide predictions of gRNAs of CRISPR/Cas9 in model plants and major crops.	Specifically designed for model crop species. It offers assessment of off-target effects based on mismatches and alignment positions. However, it should be noted that the off-target evaluation may not be as comprehensive as that provided by some newer tools, which were last updated and developed in 2014.	January 2014	Xie *et al.* (2014)
E-CRISPR http://www.e-crisp.org/E-CRISP/index.html	A web-based tool to design and evaluate CRISPR in entire genomes using fast indexing approach. This algorithm can generate genome-scale libraries for several organisms within a few hours.	This tool allows genome-wide gRNA design with a fast and accurate algorithm. It is also suitable for major cereal crops such as rice, maize, and wheat. It provides complete guideline for user by providing 'Help' page. Users can install E-CRISPR locally using Docker. The installation guideline can be accessed via https://github.com/boutroslab/cld_docker.	July 2019	Heigwer *et al.* (2014)
CCTop https://cctop.cos.uni-heidelberg.de	Online software to determine viable CRISPR/Cas9 target sites in an input query sequence. The genome references available are pretty diverse, including more than ten species of the Poaceae family. It also offers a range of analysis tools for assessing the efficiency and specificity of CRISPR-mediated gene editing.	This tool supports 17 PAM motifs, making it one of the most flexible CRISPR tool designs. It provides off-target analysis and assigns scores based on GC content and exon position, but may not be as advanced as in other newer CRISPR design tools due to its development in 2015 and 2017.	No information available	Stemmer *et al.* (2015); Labuhn *et al.* (2018)
CGAT http://cbc.gdcb.iastate.edu/cgat	A web server that offers users to identify potential target sites for CRISPR gene editing in plants.	This tool offers a pipeline for gRNA design and provides ranking for off-target analysis. It is suitable for cereal crop research such as maize and rice. However, the interface is less intuitive compared to others tool.	No information available	Brazelton Jr *et al.* (2015)
Breaking-Cas https://bioinfogp.cnb.csic.es/tools/breakingcas	A web-based application that allows users to design gRNAs for CRISPR-based experiments and comprises multiple reference genomes in the ENSEMBL database.	The tool supports multiple reference genomes from the ENSEMBL database. It allows users to install and run the tool locally, providing flexibility and making it suitable for large-scale data. However, some technical expertise is required to set up and run locally.	February 2020	Oliveros *et al.* (2016)

https://doi.org/10.55230/mabjournal.v54i4.3226

https://doi.org/10.55230/mabjournal.v54i4.3226

Table 3. Continued

Benchling https://www.benchling.com/crispr	A web-based tool for editing DNA using the CRISPR/Cas9 system that allows the development, visualisation, optimisation, and annotation of multiple gRNA sequences with fast processing time. The sgRNA design tool is part of the more extensive Benchling life science data management and collaboration platform.	This tool offers advanced features for CRISPR design for 248 reference genomes, including those of different organisms ranging from viruses to humans. It provides on-target and off-target scores for gRNA sequences, and offers visualisation tools for target genes and exons. Some features, however, require a paid subscription.	June 2020	Pellegrini (2016)
CRISPR-GE http://skl.scau.edu.cn	A web-based toolkit for researchers to design the sgRNA (targetDesign), predict off-target sites (offTarget), and generate primers for the construction of the sgRNA expression cassettes and amplification of target sites (primerDesign, DSDecode, and seqDownload).	The tool offers an integrated toolkit that covers all steps of CRISPR experiments, such as gRNA design, off-target prediction, and primer design. It also supports multiple CRISPR systems such as Cas9 and Cpf, and provides post-editing analysis tools such as DSDecode for decoding sequencing data. However, this tool is limited to a few crop species.	No information available	Xie *et al.* (2017)
CRISPR-Local http://crispr.hzau.edu.cn/CRISPR-Local	A web-based prediction tool to design high-throughput gRNAs with high efficiency. Using CRISPR-Local, users can design a gRNA based on a provided reference or a user-defined genome/source. It integrates data from whole-genome and transcriptome sequencings.	Users can provide custom reference genomes to design the gRNA. It allows users to design gRNAs for multiple genes simultaneously.	No information available	Sun *et al.* (2019)
WheatCRISPR https://crispr.bioinfo.nrc.ca/WheatCrispr	A web-based prediction tool allowing the user to browse, select, design an effective gRNA for a specific target gene, and predict the potential off-target sites.	The tool is specially designed for wheat. It uses the Doench algorithm for on-target specificity and off-target prediction, making this tool more efficient, and provides graphical outputs for easy visualisation. Since it is limited to wheat, it may not be suitable for other crops.	No information available	Cram *et al.* (2019)
PnB Designer https://fgcz-shiny.uzh.ch/PnBDesigner	A web-based application to compute prime and base editor gRNAs for animals and plants.	This tool supports prime editing, base editing, and advanced CRISPR techniques, and allows users to design single and multiplex gRNAs for multiple genes. It provides visualisation of edited bases in the output. Nevertheless, the interface may be less user-friendly compared to some other tools.	No information available	Siegner *et al.* (2021)
CRISPR-cereal http://crispr.hzau.edu.cn/CRISPR-Cereal	A gRNA-designing tool that integrates the information on putative single nucleotide polymorphism (SNP), gene expression profile, and regulome information for maize, rice , and wheat.	The tool combines SNP information, gene expression data, and regulome insights for accurate gRNA design. It is specifically tailored for maize, rice, and wheat. Additionally, it offers off-target analysis, accommodating up to five mismatches.	No information available	He *et al.* (2021)

Analysis tools for CRISPR post-editing

It is vital to perform a posterior analysis of the gene editing experiment to validate the workability of the CRISPR/Cas system and characterise the types of mutations that have occurred. Various software tools can be used to analyse the outcomes of CRISPR editing through the computational decomposition of the sequencing chromatograms. These tools can aid researchers in analysing the data generated from polymerase chain reaction (PCR) and DNA sequencing to decode superimposed chromatogram peaks and identify the changes made to the genome by CRISPR editing. These tools typically work on any species since genome data is not always required. They offer a range of features and functions, such as alignment of sequence data, variant detection, annotation, and visualisation of editing events. Here we enlist a few decent tools that can be accessed online for free and have been utilised to successfully characterise mutated sites in cereals. Other sophisticated custom scripts and commercial programmes are available, but they are beyond the scope of this review.

TIDE/TIDER

Tracking of Indels by DEcomposition (TIDE) and Tracking of Insertions, DEletions, and Recombination events (TIDER) are both freely accessible tools that can be used to analyse CRISPR-mediated editing events. These free web tools are available on the official website (http://tide.nki.nl), and their R scripts are available upon request. The TIDE is much simpler and only accepts two standard Sanger capillary sequencing reactions, while TIDER (an improved version of TIDE) requires three Sanger sequence traces (Brinkman *et al.,* 2014; Brinkman *et al.,* 2018). TIDE can quantify small indels based on control and sample chromatograms provided by the user. The features of indels are illustrated in bar graphs. For CRISPR/Cas9 editing using a repair template, the user should utilise TIDER, and the programme requires control, reference (template), and sample chromatograms as inputs to quantify templated mutations. Both tools have a simple yet informative user interface, allowing rapid processing of inputs and displaying of outputs. They are not species-dependent, as researchers have been utilising both programmes for genome editing experiments in both plant and non-plant organisms. The implementations of TIDE in CRISPR work have been reported in wheat and maize (Okada *et al.,* 2019; Baer *et al.,* 2023), while genome-edited cereals analysed by TIDER have not been reported so far.

DSDecode

In addition to the homozygous mutation, which is much easier to characterise, genome editing in diploid organisms results in uniform heterozygous and biallelic mutations on the alleles. Direct sequencing of PCR products containing such mutations will display superimposed sequencing chromatograms. The Degenerate Sequence Decode (DSDecode, http://www.ygliulab.club/dsdecode) programme decodes these sequencing chromatograms and determines the mutation type—biallelic or heterozygous—occurring in a specific region (Liu *et al.,* 2015; Ma *et al.,* 2015). It can be accessed through the web, or the user can request a personal computer (PC) version. As mentioned, DSDecode is part of the simple yet multifaceted CRSPR-GE toolkit (Xie *et al.,* 2017). Using DSDecode, the user starts by providing a reference sequence and uploading the chromatogram file. The user has to select the first overlapping peak on the chromatogram and manually generate a short degenerate sequence (DS) next to the anchor sequence (AS). Then, the programme will run to decode the superimposed peaks or any indels. Semi-automatic decoding (SaDSDecode) can also be used if the automatic decoding fails. Wang *et al.* (2016) employed the DSDecode tool to decode superimposed peaks in CRISPR-edited rice, one of the few reports demonstrating the utilisation of the web tool for cereal crop editing.

Inference of CRISPR Edits (ICE)

Inference of CRISPR Edits (ICE, https://www.synthego.com/products/bioinformatics/crispr-analysis) is a user-friendly software tool that offers a quick and reliable analysis of data produced from CRISPR editing. The ICE supports various edits, including indels, base substitutions, multi-guide fragment deletions, and HDR insertions (up to ~200 bp) (Conant *et al.,* 2022). The tool's capability to conduct batch analysis extends its function to process high-throughput datasets, wherein hundreds of edits are analysed simultaneously. This free tool is compatible with edits from any species and does not require the user to upload a reference genome. The ICE tool was successfully utilised to characterise the mutations on the *OsBADH2* and *ZmIPK* genes in CRISPR-edited rice and maize, respectively. (Ashokkumar *et al.,* 2020; Sant'Ana *et al.,* 2020). It was also employed to discern the types of mutations on *GW2-B*, *PinB-D*, and *ASN2-A* genes in mutant wheat (Brandt *et al.,* 2020).

CRISPR/Cas web-based database

Collecting validated gRNA sequences to assist researchers in choosing an effective gRNA is of great interest. In addition, the number of plant species that have been genome-edited using CRISPR/Cas9 technology has recently increased. These genome-edited plants have mutations in a wide array of genes with various roles in growth, development, and abiotic and biotic stress traits. Several efforts have been made to develop web-based databases that collect and store information on CRISPR-based experiments, such as genome-edited mutants, mutant identifiers, experiment types, and validated gRNAs (Zheng *et al.,* 2019; Zhang *et al.,* 2020). However, little information is provided about the transformation experiments, the varieties used, the progenies derived, and the seed availability in genome-edited plants, specifically in the Poaceae family. A central repository for experimental data collection from CRISPR-based experiments is urgently needed to share information on CRISPR/Cas-generated plant mutants, thereby saving time and increasing the efficiency of CRISPR-based experiments.

CRISPRlnc

CRISPRlnc (http://www.crisprlnc.org/) is a manually curated database of validated CRISPR/Cas9 sgRNAs for long noncoding RNAs (lncRNAs) from all species (Chen *et al.,* 2019). The curated data in CRISPRlnc was retrieved from more than 200 published papers and thousands of validated sgRNAs. Hence, this database provides a comprehensive list of validated CRISPR/Cas9 gRNAs for lncRNAs from all species. However, the only available plant species in CRISPRlnc is *S. lycopersicum.* This database helps the user browse and download related information on validated CRISPR/Cas9. Users can retrieve important

data, such as ID, position in the genome, sequence, CRISPR type and gRNA, and functional description of lncRNAs. It also provides third-party tools, such as BLAST for similarity search analysis and genome browser for visualisation.

PGED

The Plant Genome Editing Database (PGED, http://plantcrispr.org/cgi-bin/crispr/index.cgi) was developed due to the lack of databases to manage the data of CRISPR-generated plant mutants (Zheng *et al.,* 2019). PGED consolidates information on CRISPR/Cas-generated mutants in plant species that have been developed and reported in the published literature. The PGED allows the researchers to gain information on mutant lines, including the number of mutant plants, their generation, the type of mutations, and the observed phenotypes. It can also serve as a central repository for users to systematically manage their experimental data. This database offers information on genes and transformed rice lines that have been validated using CRISPR/Cas technology, such as primers, gene identifiers, position, experiment type, and contact person. However, for the family Poaceae, only rice is listed in the PGED. Interestingly, this database also allows researchers to submit information on genome-edited plant mutants from their CRISPR/Cas9 experiments.

Plant-Crispr

Plant-Crispr (http://www.grapeworld.cn/pc/index.html) offers an efficient database for GEd in 144 plants (Wang *et al.,* 2021). The Poaceae family in Plant-CRISPR includes rice, barley, millet, and wheat. Users can utilise search functions to find the sgRNAs using species name, gene identifier, chromosome, and position. Users can also utilise the browse function, select plants of interest, insert the gene ID, and choose the options to search potential or specific sites. The output of this database provides the GC content, predicted PAM-rich regions (PARRs), G-quadruplex (G-Q) structures, and related gene information linked to the editing site. Plant-Crispr also considers suggestions from users to share newly genome-edited plant information.

PERSPECTIVE, CHALLENGES, AND CONCLUDING REMARKS

Unsustainable development and substantial yield losses in most crops are the results of climate change, along with both abiotic and biotic stresses. To enhance the capabilities of conventional breeding techniques, GEd has been employed. This approach facilitates the development of high-yielding and high-quality crop cultivars by introducing novel genetic variations, better meeting the increasing needs of consumers. In this article, we have presented an overview of the major GEd technologies for plants and summarised the discoveries on potential genes that can be CRISPR-edited to develop important cereals with tolerance to salinity and drought stresses. These potential genes could be utilised by other researchers to enhance desirable traits in various plants, particularly in minor grain crops like sorghum, oats, and millet. Additionally, these genes could be extended to other grains—including pseudocereals—which might be less familiar but frequently play a crucial role in unique local food systems.

To the best of our knowledge, rice remains the most studied and utilised plant in the GEd experiments compared to other cereals, all thanks to the updated genome sequences, rigorous gene functional studies, and scores of improved delivery techniques of GEd systems. Therefore, the research disparity between *O. sativa* and other crops should be lessened. The CRISPR/Cas system is revolutionary for the fundamental study of gene functions and crop improvement initiatives in agriculture; hence, researchers specialising in other crops should take full advantage of the technology.

The future direction of bioinformatics in GEd could be focused on increasing the precision and efficiency of GEd prediction and minimising off-target effects by developing more advanced algorithms, predictive models, and software tools. Consequently, the advanced bioinformatics tools and software will accelerate GEd research and applications. However, these computational tools should be developed as user-friendly and web-based applications, where biologists lacking experience in computational tools will better understand the mechanism underlying GEd and be able to design more precise and effective editing strategies. On top of those, a one-stop database could be developed to store successfully edited genes and genomes to encourage data sharing and act as a focal reference for plant breeders working on genome editing.

However, a few factors of concern need to be investigated to utilise this technology to its full potential. Situations such as unwanted off-target events, ethical issues, local regulations, and public acceptance must be addressed. The off-targets can arise due to the CRISPR system's innate imprecision—mostly due to the sgRNA design—the high degree of sequence similarity in different parts of the genome, or the combination of both. To minimise the likelihood of off-target events and improve the precision of the CRISPR system, researchers have developed specialised and integrated computational tools. Even so, off-target effects can still occur, necessitating careful consideration when choosing the appropriate CRISPR/Cas versions.

Many countries welcome genome-edited crops and encourage the adoption of the new technology; others remain undecided on this matter. Spök *et al.* (2022) discuss the recent developments on GEd acceptance in selected countries, and the United States (US)—a well-known GMO-adopting country—has been lenient on this New Genomic Technique (NGT). For example, CRISPR-edited mushrooms (*Agaricus bisporus*) and false flax (*Camelina sativa*) have been exempted from biosafety regulations by the US government (Waltz, 2016; Waltz, 2018; Spök *et al.,* 2022). Australia, Canada, Ecuador, Guatemala, Honduras, India, Japan, Kenya, Nigeria, Paraguay, Philippines, Russia, and Thailand have also taken a stance to exclude certain categories of genome-edited plants from GMO category (Buchholzer & Frommer, 2023; Chanikornpradit, 2024). While several European Union (EU) countries are starting to adopt a welcoming stance towards genome editing, the United Kingdom (UK) government has eased the law for scientists to embark on plant GEd, and crop products produced through the technology will be allowed on the market without labelling requirements—a major step forward for the scientific community (Vaughan, 2022; Buchholzer & Frommer, 2023). In Malaysia, the legal stance concerning products developed using gene editing technology is still unavailable, even though regulatory frameworks in GM research and products have been well-established by the government (Kalidasan & Theva Das, 2021). As of the time of writing, any activities involving genome editing in Malaysia are required to undergo prior notification and obtain approval from the National Biosafety Board (NBB). This regulatory framework is designed to ensure that all genome editing practices are carried out responsibly, ethically, and in compliance with the Biosafety Act (2007) and the Biosafety (Approval and Notification) Regulation (2010). These varying regulations imposed by the governments largely

reflect their regulators' and citizens' acceptance, and many countries remain undecided on the utilisation of the technology. Dissatisfaction and public hesitance towards GEd crops that stem from the perception on GMOs are more directed towards safety and ambiguous product manufacturing procedures. Hence, it is critical to educate the public about the benefits and safety of NGT, particularly in countries where abiotic or biotic pressures threaten the dominant crop. To ensure that the GEd is utilised efficiently and ethically in agriculture, researchers, regulators, and the general public must continue to engage in discourse and debate to address the applications of this technology.

In general, CRISPR/Cas-based technologies will likely continue to change breeding efforts towards producing climate-resilient crops to secure the future food supply. It has tremendous potential benefits in agriculture and is expected to play a significant role in boosting crop plant production and sustainability, thus providing considerable benefits to farmers, consumers, and the environment.

ETHICAL STATEMENT

Not applicable.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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