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Genome editing in cotton: challenges and opportunities

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Abstract

Cotton has enormous economic potential providing high-quality protein, oil, and fibre. A large increase in cotton output is necessary due to the world's changing climate and constantly expanding human population. In the past, conventional breeding techniques were used to introduce genes into superior cotton cultivars to increase production and to improve quality. The disadvantages of traditional breeding techniques are their time-consuming, reliance on genetic differences that are already present, and considerable backcrossing. To accomplish goals in a short amount of time, contemporary plant breeding techniques, in particular modern genome editing technologies (GETs), can be used. Numerous crop improvement initiatives have made use of GETs, such as zinc-finger nucleases, transcription-activator-like effector nucleases, clustered regularly interspaced palindromic repeats (CRISPR), and CRISPR-associated proteins systems (CRISPR/Cas)-based technologies. The CRISPR/Cas system has a lot of potential because it combines three qualities that other GETs lack: simplicity, competence, and adaptability. The CRISPR/Cas mechanism can be used to improve cotton tolerance to biotic and abiotic stresses, alter gene expression, and stack genes for critical features with little possibility of segregation. The transgene clean strategy improves CRISPR acceptability addressing regulatory issues associated with the genetically modified organisms (GMOs). The research opportunities for using the CRISPR/Cas system to address biotic and abiotic stresses, fibre quality, plant architecture and blooming, epigenetic changes, and gene stacking for commercially significant traits are highlighted in this article. Furthermore, challenges to use of CRISPR technology in cotton and its potential for the future are covered in detail.

Keywords ZFNs, TALENs, CRISPR/Cas9, Cotton, Genetic improvement

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Background

From germination to harvesting, cotton experiences a variety of biotic and abiotic stresses. Climate change has become another havoc for cotton that has put it in threat of an unfavored environment (Uniyal and Dietrich 2019). Prolonged heat, cold and unexpected rains change the insect and disease dynamics. It also changes the scenario of abiotic factors like soil composition effecting the soil web, causing drought, salinity, and water scarcity (Onyekachi et al. 2019). All these factors integrate to make cotton a less profitable crop discouraging the farmers to grow it. Absolutely, cotton deserves more value as it is the only spendable natural fiber crop as compared to flax plant. Sustainable practices have

been introduced to increase soil health, microbial diversity and crop yield (Wang et al. 2020). A sustainable and profitable cotton production system is required to address all the problems keeping in view the sustainable development goals (SDGs) set by United Nations (Marzec and Hensel 2020).

Genome editing (GenEd) has revolutionized the field of life sciences and is used for genetic engineering in plants and animals with equal success. Researchers are using GenEd technology to get precise genetic modifications (Wen et al. 2018). Precise genetic engineering has been a longstanding fundamental goal of scientists conducting research in the field of synthetic biology, gene therapy, drug development, molecular breeding and biotechnology. The goal was achieved when it was reported that creation of targeted in vivo modifications to genomes can be successfully achieved using engineered nucleases (ENs) (Bogdanove and Voytas 2011). Basic questions in the biology and biotechnology can be addressed using available GenEd platforms. Researchers are using ENs; zinc finger nucleases (ZFNs), transcription activator like effector nucleases (TALENs), meganucleases, clustered regularly interspaced short palindromic repeats (CRISPR) / CRISPR-associated protein (CRISPR/Cas) etc., from last couple of decades to recruit repairing machinery of the cell by creation of double-strand breaks (DSBs) at predefined target sites (Durai et al. 2005; Kim et al. 1996). The binding of a reprogrammable EN to the target sequence is the prerequisite to produce a DSB at the desirable target site (Mahfouz et al. 2014).

Previously, several techniques have been used for mutagenesis in plants. Mutations in plant genomes were produced for different purposes such as biotic resistance, abiotic stress tolerance, dwarfism etc. X-ray was the first mutagen used for mutation in drosophila (Muller 1927). There are several other physical (radiation), chemical (EMS) and biological (transposable) mutagens (Stadler 1928; Greco et al. 2001; Kim et al. 2006) which have been successfully employed for mutagenesis in plants.

Mutation breeding has produced 3 200 plant varieties since 1930 to 2014 exhibiting different traits of interest. The problem with use of these mutagenesis is off-target mutation that have detrimental effects, difficult to screen out and may produce confusing results for researchers (Podevin et al. 2013). With the discovery and innovation in scientific world, new specific and efficient mutagens have also been used such as targeting induced local lesions in genomes (TILLING) technologies and recombinases. These mutagens have been widely used for reverse genetics and functional genomics studies. GenEd technique has been found fruitful in plants and animals with equal success to provide site-specific/targeted mutagenesis (Woo et al. 2015). From eukaryotic to prokaryotic organisms, GenEd tools were found marvelous in precise genome editing for different purposes (Woo et al. 2015).

Utilizing GenEd technologies, a number of organisms have already been genetically created for selective genetic modification e.g. *Arabidopsis thaliana* (Cermak et al. 2011), tomato (Brooks et al. 2014; Li et al. 2018; Bari et al. 2019), grapes (Ren et al. 2016), potato (Butler et al. 2016; Clasen et al. 2016), banana (Kaur et al. 2017; Kaur et al. 2018; Kaur et al. 2020), sorghum (Jiang et al. 2013b), soybean (Curtin et al. 2011; Jacobs et al. 2015), maize (Liang et al. 2014; Char et al. 2017), cassava (Odipio et al. 2017; Gomez et al. 2019), *Citrus sinesis* (Wang et al. 2019), Kiwi fruit (Wang et al. 2018a, b), wheat (Wang et al. 2014; Kim et al. 2018), rice (Li et al. 2012; Hu et al. 2016; Shufen et al. 2019), tobacco (Mahfouz et al. 2011; Gao et al. 2015), cotton (D'Halluin et al. 2013; Iqbal et al. 2016; Chen et al. 2017; Gao et al. 2017a; Li et al. 2017; Wang et al. 2018a, b), bacteria (Jiang et al. 2013b, a), fungi (Liu et al. 2017), yeast (*Saccharomyces cerevisiae*) (Li et al. 2011), viruses (Ali et al. 2015; Ji et al. 2015; Khan et al. 2018, 2019), drosophila (Gratz et al. 2013), mouse (Nelson et al. 2016), insects (Watanabe et al. 2017a), *Caenorhabditis elegans* (Cheng et al. 2013), zebrafish (Huang et al. 2011), rats (Tesson et al. 2011), sheep (Zhao et al. 2016), cattle (Gao et al. 2017b), goat (Zhou et al. 2017), pigs (Watanabe et al. 2017b), human cell lines (Miller et al. 2011). Advancement in gene editing technology, like base editing and prime editing which are more promising with high precision, has minimized the chance of off-targets (Mishra et al. 2020). Prime editing and base editing has been reported in wheat, rice, maize, and cotton (Biswas et al. 2022).

All ENs produce DSBs at the target site in the DNA sequence followed by repairing the cellular machinery with or without errors using non-homologous end joining (NHEJ) or homology directed (HDR) repair pathways (Rouet et al. 1994; Salomon and Puchta 1998; Bibikova et al. 2003). NHEJ and HDR are used for gene repair, replacement of faulty genes and insertion of new gene (Zhang et al. 2013). For gene insertion in site specific manner, another method, Obligate Ligation-Gated Recombination (ObLiGaRe), were experimented and introduced by.

ObLiGaRe is an additional tool used for broad applications in genetic engineering and targeted gene modifications (Yamamoto et al. 2015). ObLiGaRe can also be used for gene tagging, reporter gene insertion, purification of fusion proteins and monitoring of gene expression (Maresca et al. 2013).

CRISPR-based genetic modification can be employed to edit a gene as well as a metabolic pathway. Multiplexing allows for the simultaneous editing of 6–8 genes,

however the lower profits associated with GenEd discourage farmers from cultivating it especially in the countries where genetically modified organisms (GMOs) have social concerns (Bortesi and Fischer 2015). So, breeders involved in the development of a variety of the multigene characters can use multiplexing technology to get high accuracy and efficiency. In this review, a general description of the available genome editing platforms has been given with special emphasis on using these platforms for genetic improvement in cotton.

GenEd tools used for targeted genome modifications

ZFNs, mega-nucleases, TALENs, and CRISPR/Cas9 are famous gene editing tools which have been demonstrated for targeted gene modifications in plants (Aziz 2021). CRISPR is a well-known bacterial immune defense system with it's the best and well know tools known as CRISPR that laid the new foundation for biotechnologies on CRISPR-Cas9 (Singha et al. 2022).

These GenEd tools can target and precisely edit the DNA sequence in the genome for genetic improvement in the organism (Table 1). The designing and reprogramming of GenEd tools according to the target sequence is possible. GenEd tools such as ZFNs, TALENs, and CRISPR/Cas are different in designing, cloning/construction, expression vectors and transformation methods but are similar in the basic principle of creation of DSBs at the target site.

The wise and efficient use of DSBs depends upon the selection of genome editing technology opted for that purpose. Moreover, DSBs can be used for the creation of targeted heritable mutation in general with some Indels (insertion/deletion of DNA bases at the DSBs). Insertion, correction, and replacement of a gene are also possible using a donor template (Lo et al. 2013). But it has been found less efficient in the case of plants. Furthermore, the deletion of a gene can be achieved by the creation of two DSBs in the flanking regions of the gene.

Several on-line and off-line software are available for designing and in silico assembly of the GenEd tools. The clones/plasmids are also available from different scientists or from non-profit plasmid repositories such as Addgene. Apart from ZFNs, TALENs and CRISPR/Cas which have been used more frequently and widely for genome editing, other ENs are also available such as Meganucleases or homing endonucleases (DADGILA-GLI) which have also been reported for targeted genome modifications (Roth et al. 2012), but the applicability of Meganucleases is very limited compared to other ENs.

Zinc finger nucleases

The first targeted gene mutation was achieved in tobacco plants at the end of the previous century which involved DSBs. A natural meganuclease I-*SceI* 18-bp recognition site was used to achieve the target (Puchta et al. 1996). A selective and distinctive phenotype was reported after the repairing of DSB through homologous recombination in tobacco (Puchta et al. 1996; Puchta 1998). Targeting the specific DNA sequence in the provided genomes, ZFNs are the first extensively utilised artificial nucleases (Dong et al. 2021). *FokI* nuclease is a bacterial endonuclease which is fused with Zinc Fingers (ZFs) to create DSBs in a predetermined DNA sequence (Kim et al. 1996). ZFN-based gene targeting was first reported in animal systems (Bibikova et al. 2001). During 1990s, *Drosophila melanogaster* was the first organism targeted for ZFNs-based genome modifications (Bibikova et al. 2003).

For the creation of a DSB, a dimer of ZF monomers and *FokI* endonuclease is required. Previously, three ZFs636 recognizing 9-bp DNA binding sites were used for the successful creation of DSBs in the target DNA (Kim et al. 1996; Smith et al. 2000). Targeted mutation has been developed in *Arabidopsis* at the seedling stage through high temperature relative expression of ZFNs (Lloyd et al. 2005). It was found that 10% of the plants had desired mutations which were transmitted in the subsequent generations. The function of a defective GUS was observed after repairing via homologous recombination and by integrating the *NPTII*

Table 1 Characteristics of various gene editing tools

Characteristic	ZFN	TALEN	CRISPR/Cas9	References
Binding principle	Protein-DNA	Protein-DNA	RNA-DNA	Cui et al. (2022)
Ease of design	Moderate	Easy	Very Easy	Buljung et al. (2022)
Assembling	Difficult	Easy	Very Easy	Buljung et al. (2022), Li et al. (2022a)
Time for construction	5–7 days	5–7 days	1–3 days	Buljung et al. (2022), Li et al. (2022a)
Cost	High	Moderate	Low	Khan et al. (2022)
Efficiency	Variable	High	High	Zeng et al. (2022)
Off-target effects	High but variable	Low	High	Kovalchuk (2021)
Single-unit or pair	Pair	Pair	Single unit	Tyagi et al. (2021)

reporter gene at various chromosomal sites in 10 different transgenic tobacco lines (Wright et al. 2005). Gene targeting efficiency of ZFNs was also tested in tobacco against endogenous acetolactate synthase (ALS) genes (*SuRA* and *SuRB*) and herbicide-resistant plants were observed with allelic mutations transferable to new generations (Townsend et al. 2009) (Table 2).

In maize, an herbicide resistant gene was specifically targeted to a particular locus in several separate events and then added by the co-expression of ZFNs with a complementary donor molecule. This resulted in genetic modifications in advanced generations (Shukla et al. 2009). Gene replacement of 7-kb fragment with a 4-kb donor cassette has been successfully achieved based on HDR. The donor template composed of red fluorescent protein (RFP) and kanamycin resistance gene was flanked by two ZFN cutting site (Schneider et al. 2016). Artificial Zinc-finger Proteins (AZPs) have been used successfully for virus interference (begomoviruses) (Sera 2005; Takenaka et al. 2007). AZPs has also been used for blockage of Rep protein binding site to inhibit viral replication in begomoviruses infecting a number of plants including cotton (Mao et al. 2013). Similar strategies can be used for suppression of other viruses by targeting transcription factor (TF) binding sites in the conserved DNA sequences (Khan et al. 2017a, b). Use of AZPs and ZFNs is well demonstrated in plants as well as in animals and human cell lines for producing targeted gene modifications, but the difficulty in designing and cloning of ZFNs and their cost of production have opened the choice for choosing other GenEd tools which address existing problems (Lim et al. 2022).

TALEs and TALENs for targeted genome modifications

TALEs are released by Xanthomonas, which activate target genes to cause plant diseases. TALEs have an acidic transcription-activation domain (AD), a NLS at the C-terminus, a DBD in the middle, and signals for secretion and motility at the N-terminus (Ma et al. 2016). The 34 amino acid repeats are arranged in 14–20 tandem arrays in the core of the DBD. The amino acid sequences of the repeats are almost identical, with the exception of double residues at positions 12 and 13 which are called repeat variable di-residues (RVDs). The effector proteins' crystal structures reveal that the first RVD amino acid in position 12 stabilizes the repeat structure, while the second RVD amino acid in position 13 identifies the sense strand DNA nucleotide (Maeder et al. 2013). The letters HD, NG, NI, and NN all begin with the letter C. NK and NH are more guanine (G)-specific than NN. As long as there is a thymine (T) before the target sites initial nucleotide, TALENs may target any recognition site in the genome (Modrzejewski et al. 2019).

Making TALENs is challenging since TALEs' DBDs are lengthy and repetitive. TALEN may be assembled using a variety of techniques, including conventional cloning, Golden Gate, and solid-phase (Maeder et al. 2013). The Golden Gate ligation technique, which is less expensive, simple to regulate, and suitable for small-scale research, was used to create the majority of TALENs that target plant genes. Researchers may link up to 10 TALE repeats in a single reaction using Type IIS restriction enzymes and the same reaction mixture. The genes of tobacco, rice, *Brachypodium*, barley, *Arabidopsis* and many other plant species have been targeted with TALEs and TALENs. TALEs have been employed for suppression of cotton leaf curl virus (Khan et al. 2018).

Briefly, natural TALE proteins are secreted by plant pathogenic bacteria, *Xanthomonas* (Teper and Wang 2021). These proteins hijack plant gene expression regulation and modulate expression of disease susceptibility genes, making plants vulnerable to disease. TALE proteins are comprised of two domains: the DNA binding domain and the effector domain. In natural TALEs,

Table 2 ZFN-mediated genome modifications in plants

Sr. No	Plant Species	Genes	Gene modification	References
1	*Arabidopsis*	*ADH1, TT4*	NHEJ	Shukla et al. (2009)
2	Tobacco	*SuRA, SurRB*	NHEJ	Townsend et al. (2009)
3	*Petunia*	*mGUS*	NHEJ	Marton et al. (2010)
4	Maize	*IPK1*	NHEJ	Zhang et al. (2010)
5	Soybean	*DCL*	NHEJ	Curtin et al. (2011)
6	Tobacco	*Kan, RFP*	HDR	Schneider et al. (2016)
7	Tomato	*L1L4*	NHEJ	Hilioti et al. (2016)
8	Rice	*SSIVa*	Targeted mutagenesis	Jung et al. (2018)
9	Soyabean	*FAD2-1a, DGT28, HPTII, RPF, DCL*	Targeted mutagenesis, Gene knock in	Curtin et al. (2011), Bonawitz et al. (2019)

Table 3 Comparisons between TALEs and TALENs

Characteristics	TALE	TALEN	References
Loss-of-function mechanism	Repression of transcription	Frame shift DNA mutation	Shamshirgaran et al. (2022)
Transgenes	TALE-KRAB	TALEN	Wani et al. (2022)
Guiding sequence	DBD	DBD	Singh et al. (2022)
Required sequence information	Annotated TSS	Transcriptome	Anugraha et al. (2022)
Off-target space	Window around TSS	Genome; requires FokI dimerization	Chaudhuri et al. (2022)
Transcript variants	Only variants from the same TSS	All variants via conserved region	Li et al. (2022b

the effector domain is an activator which can alter gene expression. The effector domain to the specific DNA region based on the septicity of TALE monomers which is targeted by the DNA binding domain. Each TALE monomer contains 33–35 amino acids. The 12 and 13-positioned amino acids in one TALE monomer are responsible for the specificity of the TALE monomer to the DNA base (Mao et al. 2007). For each DNA base, different RVDs have been deciphered based on their binding affinity. Hence, for four DNA bases, initially, four RVDs were given; NI for A, NG for T, HD for C and NN for G/A. Recently, other RVDs have also been discovered such as NH for G, and it was found that it is more specific in targeting G than NN because NN had similar affinity for A and G (Liang et al. 2017).

The designing and assembly of TALEs and TALENs is comparatively more simple, comprehensive, cost-effective and time saving than ZFNs. Due to the single RVD and DNA base complementarity, the modular assembly of TALE and TALENs is very easy and can be used for broad-spectrum targeting of DNA sequences. Theoretically, any DNA sequence can be targeted using TALENs, while in case of ZFNs it was not possible (Table 3). Golden gate assembly is the fastest, simplest and cheapest strategy of cloning TALEs and TALENs (Cermak et al. 2011). Many free web-based online softwares are available for designing of TALEs and TALENs according to the DNA sequence of choice (Khan et al. 2017a, b). Several companies are providing designing and cloning services for TALEN construction on commercial basis (Khan et al. 2017a, b; Khan et al. 2018). Moreover, apart from nuclease domain, other effector domains are also available for TALEs which can be utilized to improve epigenetic marks and control gene expression. Gene repressors i.e. KRAB and gene activators VP16 and VP64 are used for modulation of gene expression, while TET1 and LSD1 are used as epigenome modifiers (Sultan et al. 2022). Owing to the high targeted mutagenesis efficiency of TALENs over ZFNs, TALENs have become more acceptable and applicable molecular scissors (Chen et al. 2013). Although ZFNs and TALENs have the same nuclease domain, *FokI* nuclease, but the binding domain is more crucial in septicity and effectiveness which ultimately resulting in a high mutation rate (Mahfouz et al. 2011; Miller et al. 2011). In comparison to ZFNs, TALENs have been used more frequently in a variety of plant species for targeted gene modifications (Table 4).

CRISPR/Cas: an RNA-guided endonuclease system

CRISPR/Cas, an RNA-guided endonucleases (RGENs) system, was emerged as adaptive immune system in bacteria (Ahmad et al. 2021a). CRISPR/Cas is the simplest and easiest system in terms of designing and cloning compared to ZFNs and TALENs. The Cas protein being part of an artificial CRISPR/Cas system is derived to target site by a single guide RNA (sgRNA). A sgRNA consists of about 20 nt in its composition and is reasonably easy to design as per the required target sequence. This gRNA is complementary to the target DNA sequence-based on Watson–Crick base pairing. In bacteria and archaea, the CRISPR/Cas9 system serves as the RNA-based adaptive immune system. *Streptococcus pyogenes* is the source of the type II CRISPR system, which includes CRISPR-associated nuclease 9 (Cas9) (Zuo et al. 2022). By inserting repeats of the viral DNA into the bacterial genome, the native CRISPR system offers resistance to viruses. Transcripts of these repeats trigger a nuclease to attack the complementary DNA from the invading virus when a bacterial colony becomes infected a second time, eliminating the viral DNA (Park et al. 2022). The CRISPR/Cas9 system can be recreated in mammalian cells utilizing the following three simple components to enable its gene-targeting ability in the eukaryotic cell: Cas9, a specificity-determining CRISPR RNA (crRNA), and an auxiliary trans-activating RNA (tracrRNA) (Li et al. 2019c). A chimeric sgRNA can also be created by fusing the crRNA and tracrRNA duplexes. The wider applications due to cost effectiveness and easiness in designing and cloning have made CRISPR/Cas a prominent technology in gene editing field of research (Fig. 1).

Table 4 TALEN-mediated genome editing in plants

Sr. No	Organisms	Genes	DNA repair type	References
1	*Arabidopsis*	*ADH1*	NHEJ	Cermak et al. (2011)
2	Tobacco	*EBE* of *Hax3*	NHEJ	Mahfouz et al. (2011)
3	Rice	*EBE (AvrXa7 and PthXo3)*	NHEJ	Li et al. (2012)
4	Rice	*OsSD1, OsBADH2*	NHEJ	Shan et al. (2013)
5	*Brachypodium*	*BdABA1, BdSPL*	NHEJ	Shan et al. (2013)
6	*Brassica oleracea*	*FRIGIDA*	NHEJ	Sun et al. (2013)
7	Barley	*PAPhy_A*	NHEJ	Wendt et al. (2013)
8	Tobacco	*SurA, SurB*	NHEJ, HDR	Zhang et al. (2013)
9	Barley	*PAPHY-A*	NHEJ	Gurushidze et al. (2014)
10	Soybean	*FAD2-1A, FAD2-1B*	NHEJ	Gurushidze et al. (2014)
11	Wheat	*MLO*	NHEJ	Wang et al. (2014)
12	Maize	*Glossy2 locus*	NHEJ	Char et al. (2015)
13	*Arabidopsis*	*CLV3*	NHEJ	Forner et al. (2015)
14	Potato	*Vlnv, ALS*	NHEJ, HDR	Clasen et al. (2016), Butler et al. (2016)
15	*N. benthamiana*	*FucT, XylT*	NHEJ	Li etal. (2016)
16	Rice	*OsMST8, OsMST7, OsEPSPS*	NHEJ	Zhang et al. (2016)
17	Sugarcane	*COMT*	NHEJ	Jung et al. (2016)
18	Maiz	*MTL*	NHEJ	Kelliher et al. (2017)
19	Peanut	*FAD2*	Gene knockout	Wen et al. (2018)

GenEd technologies have significantly modified the skills to edit genome of various cells and organisms. CRISPR/Cas9 and TALENs have reshaped the agricultural biotechnology with its high throughput and multiplexed genome engineering. A specific DNA sequence in genome of an organism is bounded by an engineered binding domain (Li et al. 2019a). Gene expression may be modulated at various levels ranging from epigenetics to posttranscription by fusion of different effector domains with the engineered binding domain.

The programmable and predictable pattern of bringing DNA, RNA and protein close together is the projecting feature of CRISPR technology. The activators and repressors to a specific DNA sequence can also be recruited via CRISPR, which can further regulate expression of genes either through CRISPR-based activation (CRISPRa) or interference (CRISPRi) (Parkhi et al. 2021). In case of suppression of a polygenic trait in plants, such as gossypol production in cotton, CRISPRi could be a tool of choice. Cotton Biotechnology Laboratory at Center of Advance Studies, University of Agriculture, Faisalabad (Pakistan) is conducting similar project in collaboration with MNS University of Agriculture, Multan, Pakistan.

The study of gene function and altering the regulatory network affiliated with these genes is a powerful approach which will be more strengthened by the use of emerging site-specific genome editing technologies. Currently, the most suitable GenEd tool is the CRISPR/Cas that can target any DNA sequence in the genome and change the phenotype of the individuals (Cong et al. 2013). The artificial CRISPR/Cas system is the copy of microbial natural adaptive immune system which work by recognizing exogenous plasmids and bacteriophages based on RNAs: trancrRNA and crRNA. The difference between the two is the optimized sgRNA which is designed to target the specific DNA sequence. HNH and RuvC are the two endonuclease domains of Cas protein. The availability of protospacer adjacent motif (PAM) region is the main requirement of Cas9 protein. The Cas proteins may have differences in PAM region requirements, but commonly used Cas proteins need 5'NGG3' along with a target sequence of 20 nt length. CRISPR/Cas system has been used efficiently for gene improvement in cotton (Li et al. 2019a, b, c; Wang et al. 2022a) (Table 5).

Mechanism of off-target effects in CRISPR/Cas9 system

The length of sgRNA is ~20–23 nt, which is critical for determining the off-target sequence in the host genome. According to some researchers, the seed sequence similarity is crucial for define on and off-targets (Saha et al. 2022).

The target DNA sequence is complementary to the first 20 or so nucleotides of the sgRNA, which are then followed by a sequence known as the PAM, which is

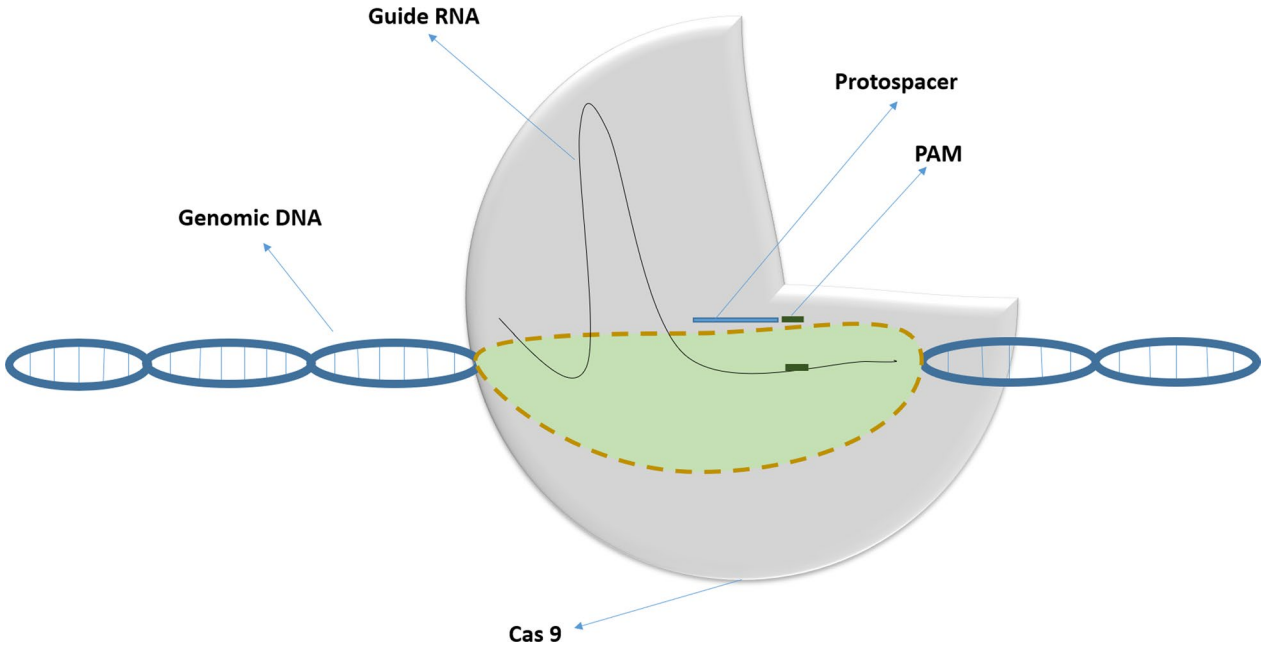

Fig. 1 CRISPR/Cas9 system for gene editing. Schematic diagram of CRISPR/cas9 system composed of sgRNA and Cas9 protein. The genomic DNA is targeted and the protospacer adjacent motif is present up stream of DNA

generally "NGG" (Slesarenko et al. 2022). Although, the 20-nt guide sequence of the sgRNA and the presence of a PAM adjacent to the target sequence in the genome are thought to tightly regulate Cas9's targeting specificity, off-target cleavage activity could still happen on DNA sequences with even three to five base pair mismatches in the PAM-distal part of the sgRNA-guiding sequence (Kang et al. 2022). At present, various CRISPR/Cas variants and types are available with different PAM requirements which may be used to enhance on-targeting. The requirement of PAM and complementary sgRNA sequence are the major factors in determining gene targeting (Li et al. 2019c).

Furthermore, earlier research has shown that various guide RNA architectures can influence the cleavage of on-target and off-target sites (Wang et al. 2022b). Crystal structure analyses and single-molecule DNA curtain experiments recommend that while the PAM site is essential for the initiation of Cas9 binding, the seed sequence corresponding to 3′ end of the crRNA

Table 5 List of some genes targeted by CRISPR/Cas9

Sr. No	Crop species	Gene editor	Target gene	DNA repair type	Target trait	References
1	Rice	CRISPR/Cas9	*LAZY1*	NHEJ	Tiller-spreading	Jiang et al. (2013b, a), Álvarez et al. (2022)
2	Rice	CRISPR/Cas9	*Gn1a, GS3, DEP1*	NHEJ	Enhanced grain number, larger grain size and dense erect panicles	Ma et al. (2015), Azadbakht et al. (2022)
3	Wheat	CRISPR/Cas9	*GW2*	NHEJ	Increased grain weight and protein content	Nahmad et al. (2022)
4	*Camelina sativa*	CRISPR/Cas9	*FAD2*	NHEJ	Decreased polyunsaturated fatty acids	Carver et al. (2022)
5	Rice	CRISPR/Cas9	*SBEIIb*	NHEJ	High amylose content	Long et al. (2022)
6	Maize	CRISPR/Cas9	*Wx1*	NHEJ	High amylopectin content	Waltz (2016)
7	Potato	CRISPR/Cas9	*Wx1*	NHEJ	High amylopectin content	Christian et al. (2022)
8	Wheat	CRISPR/Cas9	*EDR1*	NHEJ	Powdery mildew resistance	Corsi et al. (2022a)
9	Rice	CRISPR/Cas9	*OsERF922*	NHEJ	Enhanced rice blast resistance	Cromer et al. (2022)
10	Rice	CRISPR/Cas9	*OsSWEET13*	NHEJ	Bacterial blight resistance	Arnan et al. (2022)
11	Tomato	CRISPR/Cas9	*SlMLO1*	NHEJ	Powdery mildew resistance	Pramanik et al. (2021)

complementary recognition site, directly adjacent to PAM, is also critical for subsequent Cas9 binding, R-loop formation, and initiation of nuclease activities of Cas9 (Wu et al. 2022). These factors should be considered while designing a gRNA for targeting a specific sequence in the genome.

Reducing off-target effects

Designing nucleases to limit off-target effects was a critical concern not just for the fundamental research approach, but also for their potential agricultural, clinical and industrial applications. *In vivo* or *in vitro* administration of ZFNs and TALENs may result in toxicity or mortality owing to binding to off-target locations and the production of unintended DNA breakage (Jackson and Linsley 2010). In the case of ZFNs and TALENs, mutations were introduced to enhance *FokI* endonuclease activity exclusively during heterodimerization at locations bound by two distinct nucleases (Ma et al. 2015). Off-target cleavage in CRISPR-Cas9 systems is often the result of sgRNAs recognizing completely or partly complementary genomic regions. Diverse strategies have been proposed to minimize off-target cleavage, such as decreasing the quantity and duration of active Cas9 protein in cells by selective administration or modifying the half-time of Cas9 (Hajiahmadi et al. 2019). HF-Cas9, eCas9, and HypaCas9 are among the Cas9 variants with decreased off-target selectivity. New versions of Cas9 and Cas9 homologs, such as CRISPR-Cas12 (Cpf1) and CRISPR-Cas13a (C2c2), may identify alternative PAMs, which not only expands the possibilities for precise genome editing, but also has the potential for greater on-target specificity. Cas9 fusion with *FokI* nuclease is an intriguing approach that combines the benefits of ZFNs/TALENs with the CRISPR-Cas9 system (Li et al. 2019b). To mitigate off-targeting in genome editing, there is a need to focus on the in-silico studies and designing of the gene editing tools. Genome-wide off-targeting analysis should be run before going to the cloning and transformation of the gene editing tool. In case of CRISPR designing, new artificial intelligence models such as deep learning/machine learning may be adopted. A Deep-RPA model has been designed to determine the vulnerability of the genome for off-targeting (Saddique et al. unpublished data). Another strategy to minimize or avoid off-targeting is the selection of a delivery method. Various researchers used mRNA or proteins (ribonucleoprotein RNPs) and delivered them into the cells for targeted mutations to avoid integration of the transgene into the host cell (Gao 2019). Moreover, use of nano-particles has also been reported for enhancing the delivery and efficiency of transformation while avoiding the off-targeting.

Different types of CRISPR/Cas system

Cas12a (Cpf1)

Type V Cas12a is categorized as a Class 2 CRISPR system since it is comparable to Cas9 in that it simply relies on RNA molecules to create DSBs (Zhan et al. 2021). However, it simply needs a crRNA molecule to direct it to its target, in contrast to Cas9's dual guidance of a crRNA and a tracer RNA; also, the resultant DSBs are staggered cuts with 5-nt 5′-overhangs instead of the blunt cuts produced by Cas9. Additionally, whereas Cas9 enzymes recognize PAMs with G-rich sequences, Cas12a prefers to attach to targets with T-rich PAM sites. Recently, this spectrum of recognized PAMs has grown as a result of manufactured Cas12a variants (Wang et al. 2021b) (Table 6). Other advantages of Cas12a over Cas9 include its lower mismatch tolerance, which lowers off-target effects, and its ability to process its own crRNA through RNase III activity, which facilitates multiplex gene editing. This is possible because Cas12a can deliver a single pre-crRNA template to the cell, where it is then cleaved by Cas12a into various crRNA molecules that target various genes. The overhangs created when Cas12a cuts the target DNA, which helps HDR since staggered cuts are better mended using this method than NHEJ (Sledzinski et al. 2021). AsCpf1 and LbCpf1 from *Acidaminococcus sp.* BV3l6 and *Lachnospiraceae* bacteria ND2006 respectively, exhibit comparable on-target efficacy to SpCas9 in human cells (Lyu et al. 2021).

Table 6 Characteristics of different types of CRISPR systems

Characteristics	Type I	Type II	Type III	Type IV	Type V	Type VI	References
Effector complex	Multisubunit (Class 1)	Single unit (Class 2)	Multisubunit (Class 1)	Multisubunit (Class 1)	Single unit (Class 2)	Single unit (Class 2)	Zhuo et al. (2021)
Signature Protein	Cas3	Cas9	Cas10	Csf1	Cas12	Cas13	Wada et al. (2022)
Target molecule	DNA	DNA	RNA/DNA	DNA	DNA	RNA	Niu et al. (2021)
Details	Cleaves ssDNA strands	Originates blunt DSB	Binds to nascent RNA molecules	Most unknown CRISPR system	Originates staggered DSB	RNA-guided RNase	Gong et al. (2021)

Cas13a (C2c2)

The most recent member to the CRISPR family is significantly distinct from its predecessors. Although, Cas13a is a Class 2 CRISPR system, it can only cleave RNA attributable to the activity of two HEPN domains, in contrast to Cas9 and Cas12a's capacity to cut DNA (Wang et al. 2021a). It shares with Cas12a the capacity to process its own crRNA, allowing several loci to be targeted with a single pre-crRNA template. Cas13a's RNA-cleaving characteristics may be used for post-transcriptional suppression with similar efficacy to RNA interference (RNAi) techniques of RNA silencing, but with greater specificity and the capacity to cleave nuclear transcripts, which is limited with RNAi (Sun et al. 2022). Due to alternative splicing, the transcription of single DNA sequence generates several splicing isoforms, hence targeting DNA with CRISPR systems affects all mRNA isoforms (Hernandez et al. 2022). Cas13a enables the investigation of a single isoform's function or interference with its impact without affecting the activity of the other isoforms. Cas13a may also target pre-mRNA, which can be advantageous in disorders caused by incorrect splicing since the enzyme can intervene before the error develops (Sahin et al. 2021). However, Cas13a exhibited a capacity to cleave RNA without discrimination, which might limit its therapeutic use. A recent research observed no similar effects when the LwaCas13a form of *Leptotrichia wadei* was applied to mammalian cells, indicating that this collateral impact may be missing or undetected in eukaryotic cells (Deol et al. 2022).

nCas9

In conditions when gene knockouts are not preferred, the NHEJ mechanism serves no function other than to impede the preferred HDR mechanism's ability to repair DSBs (Schubert et al. 2021). Similar as before, a Cas9 nickase variant (nCas9) is generated by inserting a particular mutation into the RuvC domain of Cas9. nCas9 nicks the target DNA, creating single-stranded rather than DSBs. Single-nick prefers repaired by base excision repair, hence nCas9 may be utilized to increase the efficiency of the process by decreasing the frequency of Indel mutations arising from undesirable NHEJ repairs. Additionally, nickases may be used to improve the specificity of Cas9-directed genome editing. Scientists designed a twofold nicking method with a pair of nCas9 targeting opposing strands and adjacent gRNA targets offset by a predetermined number of base pairs (Jin et al. 2013). The coupling of nCas9 systems generates DSBs with gRNA-defined overhangs, which may lead to highly targeted gene edits when paired with HDR or initiate precise deletions in key alleles through NHEJ (Zhu et al. 2022). Even if one of nCas9's nicks is off-target, the resultant nick is readily repaired by high-fidelity base excision repair, in contrast to wild-type Cas9, where blunt off-target DSB might result in unwanted changes when repaired by the NHEJ pathway (Möller et al. 2022). This technique has the disadvantage of needing the concurrent creation and delivery of two different gRNA molecules.

dCas9

When both RuvC and HNH catalytic domains of Cas9 are modified through two silencing mutations, the system loses its DNA cleaving capabilities but retains the ability to bind to targeted sequences (Wang et al. 2022b). Researchers have demonstrated that this catalytically inactive variant of Cas9 (dCas9) can hinder transcription on its own, presumably by either blocking the pairing between RNA-polymerase and promoter sequences targeted with dCas9 or instead by halting the elongation step if the target sequence is part of an open reading frame region (García-Castillo et al. 2021). The dCas9 system can be further modified in several ways, such as fusing dCas9 to direct or indirect transcription activators (such as VP64), to increase the expression of a specific DNA sequence; or transcription repressors (such as KRAB), to increase the efficiency of dCas9-mediated transcription inhibition (Dong and Ronald 2021). The modification of genetic expression by dCas9 is a transient process, as it does not cause permanent modifications to the genomic DNA. However, specific and long-lasting modifications to genetic expression are possible through the fusion of epigenetic modifiers to dCas9 (Rahman and McGowan 2022). Several effector domains may be fused with dCas9 DBD to get various modifications in gene expression at different levels. Khan et al. (2019) used dCas9 to inhibit cotton leaf curl virus and reported that dCas9 may be used as DNA binding protein to modulate gene expression and inhibit replication of the virus in host cell.

ESpCas9, SpCas9-HF1, and HypaCas9

The interactions between Cas9 system and bound DNA strands can be changed in a different ways to improve the specificity of CRISPR targeting. Slaymaker and Gaudelli (2021) thought that Cas9 cleavage might work better if the separation between the target and non-target strands was stable. This would mean that weakening this separation in unwanted targets would reduce off-target effects. Stable strand separation is maintained

after *Streptococcus pyogenes* Cas9 (SpCas9) binds to the target site through two types of interactions: the binding of gRNA to the target strand and the formation of a positively-charged groove as a result of an unintended interaction between the HNH and RuvC domains and the negatively-charged non-target strand (Slaymaker and Gaudelli 2021; Nierzwicki et al. 2022).

Re-hybridization between the target and non-target strand is facilitated by weakening contacts on the non-target strand by lowering positive charges. As a result, off-target effects are decreased because careful base pairing between the target DNA and gRNA is necessary to establish a stable division between the target and non-target strands (Michel et al. 2021). Two "enhanced specificity" SpCas9 variants (eSpCas9 and eSpCas9) were created by engineering SpCas9 mutants with a single positively-charged amino acid residue substitution to weaken groove interactions. These variants had similar on-target efficiency to WT SpCas9 but significantly lower levels of off-target cleavage (Donohoue et al. 2021).

Kleinstiver created the high-fidelity SpCas9-HF1, a variation that resulted in undetectable genome-wide off-target cleavage, by concentrating also on the binding between Cas9 and the target region. However, Kleinstiver and his colleagues altered four SpCas9 residues that created hydrogen bonds with the phosphate backbone of the target strand instead of destroying the non-target strand contacts. This impaired gRNA binding to DNA targets in the presence of any mismatches (Corsi et al. 2022b). Alanine substitutions in all four residues originated SpCas9-HF1, which along with eSpCas9 also showed comparable on-target activity with WT SpCas9, without impactful off-target effects (Garrood et al. 2021) (Fig. 2).

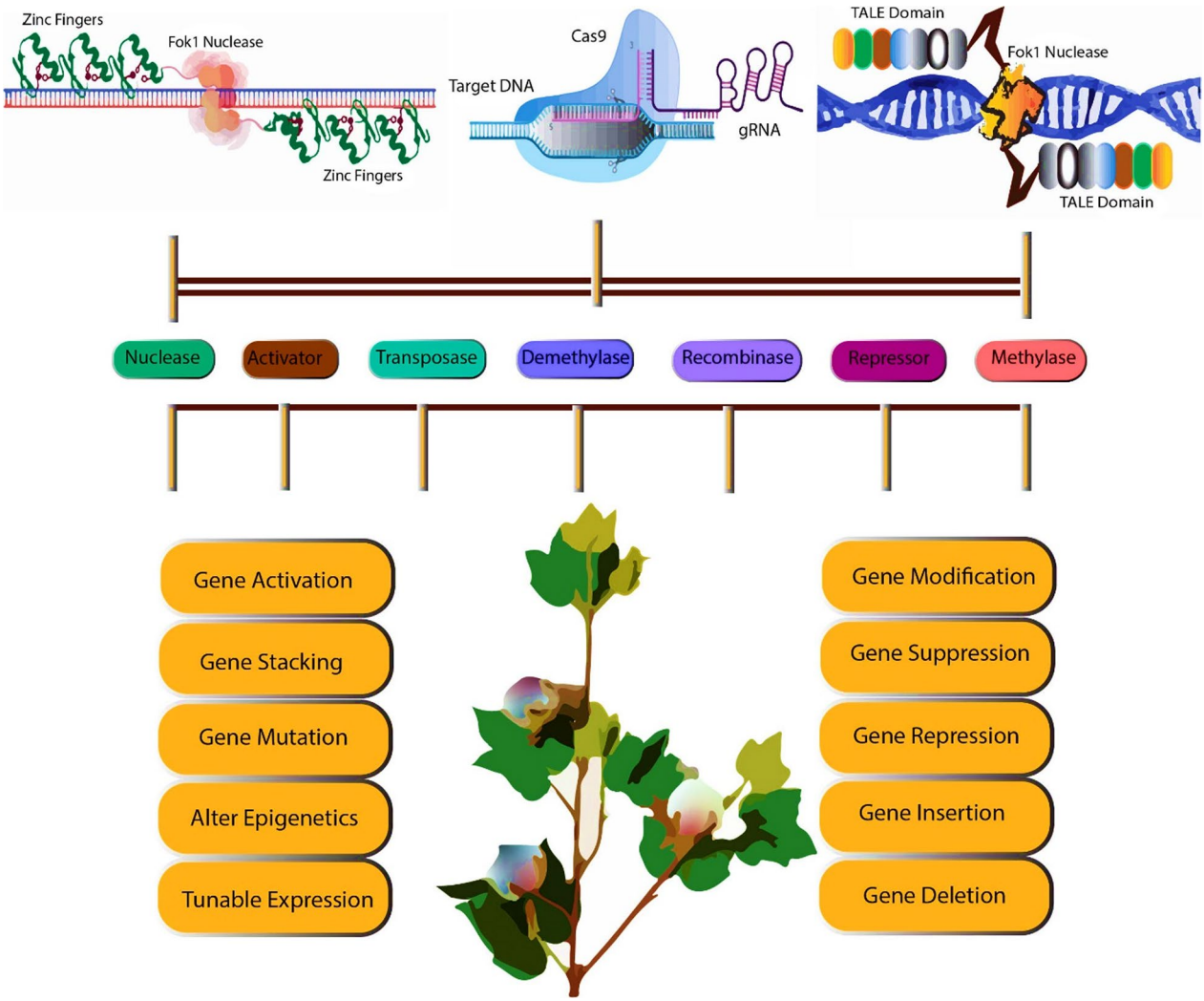

Fig. 2 Effector domain engineering with GenEd tools for different purposes. Fusion of ZFs, TALEs and CRISPR/dCas9 is possible with different effector domains for targeted gene editing and epigenetic modifications

Using GenEd tools for abiotic stress tolerance in cotton

Abiotic stresses are unfit climatic/edaphic conditions that irregulate the homeostasis or normal function of an organism, which ultimately effects its fitness and growth including plants (Schmidt et al. 2018). High temperature, drought and salinity are the major factors of cotton output reduction cross the globe and cause yield loss up to 50% worldwide (Bita & Gerats 2013). Abiotic stresses resistance are the result of the interplay of several genes and their regulatory, signaling, and metabolic pathways components, and the interaction of these components lead to response/adaptation to abiotic stress (Nakashima et al. 2009; Hirayama and Shinozaki 2010; Mickelbart et al. 2015). Several genes, transcription factors, *cis* elements and interplay of these with each other decide the fate of plant towards abiotic stress responses. Whole genome duplication events may occur in case of some abiotic stresses (Panchy et al. 2016), which may also result in functional redundancy in multi-gene families (Jain 2015). So, it is difficult to fix these multigene traits through conventional techniques to harness the resistant in plants against abiotic stresses.

Understanding of molecular basis and tolerance mechanisms towards abiotic stresses (including salinity, water deprivation, and high temperature) is critical to develop abiotic stress tolerant genetically engineered plants. There are several transcriptional factors which can be utilized as potential candidates to enhance the tolerance in cotton against drought stress (Li et al. 2013). The role of several transcription factors like ERF, NAC, MYB, WRKY and bZIP has been reported in drought tolerance as well as in normal plant development. The functional genomic studies have been carried out by cloning and validating the function of these transcriptional factors in cotton as well as in other plants. The editing of these genes has led towards the activation of several pathways in cotton critical for drought tolerance. In a previous study, *GhABF2*, a bZIP transcription factor gene, has been found to have role in drought and salinity tolerance and reported in both *Arabidopsis* and cotton (Peng et al. 2021). The role of *GhABF2* in abscisic acid (ABA) regulation was confirmed through transcriptomic analysis and higher enzyme activities of superoxide dismutase (SOD) and catalase (CAT) due to overexpressing *GhABF2* were observed in transgenic cotton plants resulting in better phenotype and yield (Liang et al. 2016).

The tolerance to abiotic factors can be effectively utilized by efficient stacking of these genes/transcription factors in modern cotton genotypes with inclusion of constitutive/strong promoters. It has been reported that overexpression of *GbMYB5* is involved in drought stress tolerance in tobacco and cotton reducing water-loss through stomata and showing hypersensitivity to ABA (Chen et al. 2015). Moreover, it is observed that sucrose non-fermenting1-related protein kinase2 (*GhSnRK2*) is positively correlated for tolerance to low temperature and high drought when this gene was silenced through virus induced gene silencing (VIGS) in plants (Bello et al. 2014). Furthermore, silencing of cotton *PHYA1* genes through RNAi increased the rate of photosynthesis and improved the root systems in plant, resulting in drought, heat and salt tolerance (Abdurakhmonov et al. 2014). Similarly, there are many genes such as *GhPIN1*-3 and *GhRDL1* that can be targeted for drought tolerance in cotton (He et al. 2017; Dass et al. 2017). Many genes of transporters, transcription factors and different enzymes such as CIPK, MYB, NAC, LEA, WD40, CDPK and NHX have been reported for reported for salt tolerance in cotton (Sun et al. 2018). There are some genes such as *IAR3*,

Table 7 Successful reports of genome editing in cotton

Sr. No	Genome editing tools	Genes	Gene modification	References
1	Meganucleases	*HPPD, EPSPS*	HDR, Gene stacking	D'Halluin et al. (2013)
2	CRISPR/Cas9	*GhPDS, GhCLA1, GhEF1*	GenEd	Cai et al. (2017)
3	CRISPR/Cas9	*GhCLA1, GhVP*	NHEJ	Chen et al. (2017)
4	CRISPR/Cas9	*GFP*	NHEJ	Janga et al. (2017)
5	CRISPR/Cas9	*GhCLA1*	Multi-site GenEd	Wang et al. (2017a, b)
6	CRISPR/Cas9	*GhARG*	NHEJ	Wang et al. (2017a, b)
7	CRISPR/Cas9	*GhMYB25-like* A & D	NHEJ	Li et al. (2017), Li and Zhang (2019)
8	CRISPR/Cas9	*GoPGF*	NHEJ	Janga et al. (2019)
9	CRISPR/Cas	*GhCLA, GhPEBP*	GenEd	Qin et al. (2020)
10	CRISPR/LbCpf1	*GhCLA1*	GenEd	Li et al. (2020)
11	CRISPR/Cas12b	*GhCLA*	GenEd	Wang et al. (2020)
12	CRISPR/Cas	*Male sterility*	NHEJ	Ramadan et al. (2021)
13	CRISPR/Cas	*MIR482 family*	NHEJ	Zhu et al. (2022)

FPGS$_3$and two ESTs (*GhHS126* and *GhHS128*) which were reported for heat tolerance in cotton (Demirel et al. 2014). GenEd tools may be used efficiently to mutate or suppress gene at transcriptional level. Multiple members of gene families may also be targeted using multiplex CRISPR system (Ahmad et al. 2021a). The success stories regarding the cotton gene editing have been given in Table 7.

There are many other plant species in which the application of genome editing has been witnessed for the improvement of abiotic stress tolerance. *SIMAOK3* mutants were developed through CRISPR/Cas9 system to study drought stress in cotton plants (Wang et al. 2017a, b). By silencing 1-aminocyclopropane-1-carboxylic acid synthase 6 gene in the transgenic maize plants showed reduced levels of ethylene biosynthesis, and grain yield was significantly improved under drought stress conditions (Habben et al. 2014). Similarly, decreasing the sensitivity of maize to ethylene also resulted in higher yield (Shi et al. 2015). ARGOS genes, negative regulators of the ethylene response, were over-expressed to enhance drought tolerance in transgenic maize plants (Shi et al. 2015; Guo et al. 2014). Mutation produced through CRISPR-Cas9 in *OsDST* gene of rice increased the salt and drought tolerance by increasing the width of leaf and reducing stomatal density (Kumar et al. 2020). The mitogen-activated protein kinase 3 (*SlMAPK3*) gene was identified by CRISPR/Cas9-based mutations as a mediator of drought defense systems in tomato plants. Rice variants produced using CRISPR/Cas9 were utilized to investigate the role of stress/ABA-activated protein kinase2 (SAPK2) in response to stress in rice. To investigate the role of C-repeat conditional factors (CBFs) in *Arabidopsis* plant cold stress response, we employed CRISPR/Cas9 technology to create cbf1, 3 dual and cbf1, 2, 3 (CBFs) triple mutants. If a geneticist understood how genes work, he or she may be able to employ genetic markers to develop more resilient crops (Zhang et al. 2021). Gene editing utilizing the CRISPR/Cas9 system may reduce the damage caused by abiotic stressors, such as high heat, dehydration, salt, nutrient insufficiency, and high levels of toxic substances.

Abiotic stresses resistances are controlled by multiples genes and several regulatory networks are involved along with signal transduction and up and down production of metabolites. These genes can be targeted through CRISPR-Cas9 technologies for inclusion of stress tolerance and crop improvement in abruptly changing climate scenarios (Ahmad et al. 2021b). The more advanced strategy could be the HDR-mediated gene targeting for the stacking and pyramiding of multiple genes at a time. Technology advancements such as base editing in CRISPR/Cas9 technology opens new endeavors of abiotic stress tolerance in plants through precised point mutation (Mishra et al. 2020). In rice, the tolerance against submergence is switched through a cytosine base-editor by altering the C>T in *Sub1A* gene (Bhowmik et al. 2019). Different genes discussed above coupled with different promising genome editing techniques can be used to equip the cotton crop with abiotic resistance/tolerance traits. Numerous abiotic and biotic factors influence agriculture all around the globe. A growing global population, food instability, and environmental pollution have prompted farmers to explore for new ways to boost yields, quality, and resistance of crops (Xu et al. 2020a). As a new method of improving agricultural varieties, CRISPR/Cas9 might be employed for functional genetic investigations. This technology might be used to improve a wide range of aspects of plant breeding in the future (Zhu et al. 2018).

Genes modifications for yield traits

Flowering in several agricultural plants is governed by seasonal variations in day duration, which may restrict the geographic distribution of cultivation for certain crops (Zhang et al. 2018). By manipulating flowering alleles and their linkages, it is possible to control blooming time. The CRISPR/Cas9 approach for editing genes like *FLOWER Genomic* and *SELF-PRUNING 5G* has already resulted in considerable modifications in the blooming time of soybeans and tomatoes (Xu et al. 2020b; Soyk et al. 2017). The thermo-sensitive genetically male sterility (TGMS) strain is one of the most often used male infertility strains in the two-line hybridization mating procedure.

TMS5 is a line of thermos-responsive, genetically engineered male sterile mice. CRISPR/Cas9 technique was demonstrated to be capable of accelerating high-yielding rice production by developing 11 new TGMS lines in just one year. The CRISPR/Cas9 approach proved successful in modifying four yield-enhancing genes: *DEP1*, *Gn1a*, *IPA1*, and *GS3*. Dep1 and gn1a cultivars had more seeds per panicle, as well as higher grain products per panicle (Gao et al. 2020). Using the CRISPR/Cas9 technology, researchers were able to alter profitability genes in farmed crop kinds with relative ease. Recent studies have shown that the CRISPR/Cas9 system may be utilized in rice to eliminate a key gene that regulates the manufacture of strigolactones. High tillering and dwarf phenotype were common in the *ccd7* mutant rice plants. Aside from that, certain CRISPR/Cas9 mutants have useful traits that may be exploited to breed and generate desired crops (Chen et al. 2021).

These genes are associated with phenotypes as varied as dwarfism and diminished fruit dehiscence in the *Brassica oleracea* species, valve-margin development in polyploid oilseed rape (*BnALC*), grain dormancy in barley (*Hordeum vulgare*), and chloroplast maturation in cotton (*GhCLA1*) (Marzec and Hensel 2020).

Gene modifications to improve the quality of products

Furthermore, through using CRISPR/Cas9 system, a single dominant *Waxy* gene controlling amylose content was knocked out in two rice varieties, and the resulting mutants showed low amylose levels and elevated glutinosity. This research provides a simple and successful method for transforming a low-quality rice variety into a higher-quality one. Furthermore, the *GBSS* gene, which encodes a granule-bound starch synthase, was damaged in tetraploid potato using CRISPR/Cas9 (Lei et al. 2021). Only lines with mutations in all four *GBSS* alleles showed a decrease in GBSS enzymatic activity. These lines had a lower amylose concentration and a higher amylopectin/amylose ratio. To improve the quality of polyploid crops, the capacity of CRISPR/Cas9 to mutate several genes simultaneously offers a straightforward and robust tool.

Genome modification to develop biotic stress resistance

EDR1 has previously been identified as down-regulation of Powdery mildew susceptibility in *Arabidopsis*. Three *EDR1* homologs were simultaneously knocked out using CRISPR/Cas9, resulting in Taedr1 plants that are more susceptible to Powdery mildew. Cas9-guided RNA-directed Cas9 knockouts for wheat and tomato improved their Powdery mildew resistance by mutating a gene called *MLO*. Citrus canker is caused by the bacterial pathogen *Xanthomonas citri* subsp. citri, which causes the canker susceptibility gene *CsLOB1* to be expressed in susceptible plants. CRISPR/Cas9 was used to alter the *CsLOB1* gene in grapefruit Duncan (*Citrus paradisi* Mac.), resulting in citrus variants that are resistant to canker. Many distinct hosts show recessive tolerance to the eIF (eukaryotic translation initiation factor) genes. CRISPR/Cas9 was successful in engineering virus-resistant cucumber and *Arabidopsis* plants by targeting genes. CRISPR/Cas9 was used to delete the *eIF4G* gene, which regulates the recessive rice tungro spherical virus (RTSV) susceptibility trait, to generate RTSV-resistant rice cultivars (Rathore et al. 2020).

Generating transgene-free and genome-edited crops

The ease, accuracy, and effectiveness of CRISPR/Cas9-induced genomic engineering and its capacity to make transgene-free, genetically engineered crops have all attracted great interest. It is possible to screen for mutant progeny plants that still carry the Cas9/sgRNA transgenes, even if they have been delivered into plants as transgenes via the CRISPR/Cas9 system. Plants that are genome-edited and devoid of transgenes are difficult to identify from those that have been mutated naturally. Industrial use of CRISPR/Cas9 may be able to avoid the stringent biosafety regulations that are required for genetically engineered food crops. In the United States, biosafety regulations for anti-browning fungi *Agaricus bisporus* and waxy corn developed with CRISPR/Cas9 were met, among several instances.

Mutant libraries construction

The task of critically analyzing the functions of all the genes in a plant genome that has been sequenced is significant. This problem can be solved by creating a genetic library that is saturated with mutants. To fine-tune the CRISPR/Cas9 system's ability to target certain genes, we changed the sgRNA's 20-bp target-binding region. Genome-wide mutations and the forward genetic testing may be carried out utilizing CRISPR/Cas9, which is both feasible and affordable. This discovery paved the way for the rising screening of plant mutant libraries using CRISPR/Cas9 in human cultured cells. When converting pooled sgRNA libraries into tomatoes, for instance, a variety of mutant strains were created. Using large-scale genetic screening and decoding, a homolog of an *Arabidopsis* boron outflow carrier gene and a gene related to immunity-associated leucine-rich repeat subclass II was swiftly revealed. Additionally, two separate research groups have developed rice CRISPR/Cas9 mutant libraries, each of which has generated a substantial number of losses of function mutations by the transformation of sgRNA libraries.

Gene transcription or translation regulation

Multiple mechanisms exist for controlling the expression of a gene's product. Plant breeding relies heavily on manipulating gene expression to promote phenotypic variation. In earlier investigations, *cis*-regulatory factors in the gene activation loop were linked to agricultural species of plants' development, domestication, and selection (Wang et al. 2022b). In tomatoes, CRISPR/Cas9 has recently been used to change the regulators of three genes associated with plant structure, inflorescence branching, and fruit size. Several promoter alterations indicated increasing variability in the *trans*-regulatory genes produced for each condition evaluated. When these transgenic crops are grown in the ground, they displayed decreased plant height, modified the color of the leaves, and increased the tiller angle. With help of the CRISPR/Cas9 mechanism, plant upstream open reading frames (uORFs) have been altered to produce higher amounts of protein, which were then transcribed into four different variations.

Gene stacking using GenEd tools

Using recombinases for genetic manipulation is an older method. Recombinase technology has been applied to site-specifically implant, remove, or reverse a target gene. It has been proposed that using site-specific recombinase technologies for gene deletion is a good way to modify genes (Andrés and Coupland 2012).

With the progress in GenEd tools like TALE proteins, TALE Recombinases (TALER) were developed by fusing TALE with DNA invertase Gin's catalytic regions (Mercer et al. 2012). TALERs have been used in mammalian cells and bacteria for targeted gene modifications. Engineered ZFs can be used as substitute of DNA binding domains to retarget the sequence of interest in the genome. These variants are the members of known resolvase/invertase family which categorically comes under serine recombinases. However, ZFNs had some hurdles like lacking in bonding with all DNA triplets, defective modularity with particular domains and difficulty in construction limited the wider application ZFPs for genome editing (Jin et al. 2013). Targeting capacity and potential applications on recombinases may be improved by TALER architecture which will be helpful for its uses in animal and plant biotechnology. In case of cotton, meganucleases have been used for pyramiding of genes based on homologous recombination (D'Halluin et al. 2013). Other efficient GenEd tools may be very useful for gene stacking because the advantage over recombinases technology is its specificity and targeted fashion even at the first event of gene integration in the host plant genome.

Future perspectives

The study and innovation in the field of genome biology and genetic modification have always been of a great interest. Development of stress tolerant and disease resistant varieties and establishment of animal gene expression in plants are the marvelous achievements of genetic engineering. Plant breeders are always eager to find variations that can be used in the breeding programs. By the advent of genome editing tools, targeted genome modifications have become possible (McGarry et al. 2013). The flexibility of using different engineered proteins and nucleases to get desirable and precise results has increased the canvas of applicability of genome editing tools. Scientists are working on the understanding and nature of genome modification tools to address limitations related with their usage. The most important limitation is the off-targeting. To address off-targeting, one can choose a different tool from the toolbox. Researchers have found that TALENs, having a long target site, have fewer off targets compared with ZFNs and CRISPR/Cas (Wang et al. 2015). The United States Department of Agriculture (USDA) has said that there would be no regulation for ENs-based precise deletions in the genome. This comes as part of the discussion around the regulation and adoption of genome edited organisms (GEOs). The scientists who are now working in this subject have expressed their optimism over this new breakthrough. Numerous agricultural plants and animals have been targeted using ENs or artificial DNA-binding proteins, and the findings have been found to be very encouraging. The toolbox that is utilized for GenEd has become more diverse, which further expands the variety of applications that can be accomplished through genome editing.

It has been shown to be more intriguing than previously existing technologies such as RNAi to suppress genes at the DNA level by introducing deletions or insertions in the target DNA. The mutations produced by using GenEd tools are more exact, specific, and efficient, and they provide outcomes that are more predictable than those produced by using other approaches such as RNAi, TILLING, and the use of other mutagens. In addition, the use of GenEd tools has made it possible to regulate gene expression in a manner that is both tunable and under remote control. Efficient regulation of the expression of native genes is possible with the use of TALEs, ZFs, and dCas either on their own or combined with effector domains. Researchers have also shown that subsequent generations of transgenic plants may be created devoid of these proteins via the process of segregation following the transformation of GenEd reagents. Therefore, these techniques may also be employed for the generation of plants that do not contain any transgenes, as well as for clean gene technology. The scientists and researchers who are working in the area of genome editing are quite eager and hopeful about the bright characteristic that this discipline has (Eş et al. 2019). These technologies are now being used across the board in the biological sciences to achieve desired genetic modifications in animals and plants.

The use of GenEd techniques in the genetic engineering of cotton will open up new possibilities for functional genomics researches, which may be used to better understand complicated metabolic processes that include several genes. By making use of the resources made available by GenEd, it is feasible to improve not only the quality of cotton fibre but also the quality of its seeds. The effectiveness of targeted gene alterations in cotton is shown by the reports of genetic engineering that were examined above. It is possible to use the CRISPR/Cas system with the nickase enzyme, which is used for gene repair and replacement, to replace an endogenous promoter with a constitutive, strong, and inducible exogenous promoter.

This will be beneficial in controlling the expression of an endogenous gene. These types of approaches are further useful to eliminate the risk of foreign gene insertion in the host plant genome. Gene pyramiding/stacking is another

tremendous feature which may be used to mitigate the segregation of desirable genes (Rathore et al. 2020). Additionally, the stacking of genes for numerous reasons, such as the enhancement of insect resistance, herbicide resistance, disease resistance, yield, and quality, would be highly desirable for the agricultural business as well as farmers. Epigenome marks associated with flowering, stress resistance, and fiber quality can be modified using ZFs, TALEs, and dCas9 with multiple effector domains. In conclusion, GenEd toolbox is helpful in solving constraints resulting in decline in cotton growth, fiber quality, and yield.

Another feature of GenEd tools is the production of DNA-free gene edited plants. For this purpose, various researchers have used different reagents ranging from delivery of DNA, mRNA, and proteins. Moreover, several delivery methods have also been reported where nanoparticle-mediated delivery may be more efficient. In case of Cas9, RNPs have been delivered to engineer multiple genes in wheat (Liang et al. 2017). Producing genome edited crops in a DNA-free fashion could be a solution to the concerns associated with the use of GMOs. Hence, genome modified cotton is already cultivated over 80% of the cultivated area, making non-GMOs gene edited cotton will be accepted globally. So, the future of CRISPR-edited crops is bright and may be helpful in addressing the important issues such as food security and sustainability.

Conclusions

Genome editing is booming. Editing nucleases have revolutionized genomic engineering, making mammalian genome editing straightforward. Since their discovery, gene editing has advanced greatly. Each of the four primary nucleases used to cut and modify the genome has benefits and downsides, and the decision depends on the context. Current genome editing methods have drawbacks, and it's tough to modify low-transfection cells or primary cultivated cells. Genotoxicity is an inherent concern of nucleic acid-acting enzymes, although highly specialized endonucleases should diminish or eliminate it. Future efforts have to be made to complement and innovate present techniques. Gene editing research should progress greatly. With next-generation sequencing technology, new clinical applications will be presented, such as creating designed medicinal items, eradicating human genetic illnesses, and treating AIDS and malignancies. Combining genomic alterations caused by targeted nucleases with self-degrading, self-inactivating vectors may assist in overcoming restrictions to enhance genome editing selectivity, notably off-target modifications. Off-target effects are still poorly understood. If CRISPR/Cas9 is to live up to its promise, more researches in this area are essential. The lack of universal gene cargo delivery mechanisms continues to be the biggest barrier to the widespread usage of CRISPR/Cas9. Since genome engineering and regenerative medicine are still in their infancy, it is necessary to thoroughly understand the functional landscape of stem and progenitor cells in various genetic contexts in order to realize the full potential of these technologies in reprogramming the destiny of these cells. Only time will tell what potential these technologies will have for humanity. One important concern is whether the immune system would recognize or reject the alien genetic components found in the cells. The fact that bioethical concerns and legal issues associated to this issue are continuing to grow in light of the possibilities of manipulating human genetic material and the riskiness of the processes involved is another significant cause for worry. Technical and ethical regulations, as well as laws, should be evaluated and need significant consideration as soon as feasible.

Abbreviations

ZFN	Zinc finger nuclease
TALEN	Transcription activator like effector nucleases
CRISPR	Clustered regularly interspaced palindromic repeats
GE	Gene editing
DSB	Double stranded breaks
HR	Homologous recombination
NHEJ	Non-homologous end joining
ObLiGaRe	Obligate ligation-gated recombination
HDR	Homology directed repair
OPEN	Oligomerized pool engineering
EN	Engineered nucleases
MN	Meganucleases

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Author contributions

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Competing interests

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