Turkish Journal of Agriculture and Forestry

http://journals.tubitak.gov.tr/agriculture/

Review Article

Turk J Agric For
(2021) 45:

doi:10.3906/tar-2012-64

Reforming cotton genes: from elucidation of DNA structure to genome editing

Muhammad Salman MUBARIK[1], Sajid MAJEED[2], Sultan Habibullah KHAN[1], Xiongming DU[3], James E. FRELICHOWSKI[5], Lori HINZE[4], Muhammad Tehseen AZHAR[2,5,*]

[1]Centre of Agricultural Biochemistry and Biotechnology, University of Agriculture, Faisalabad, Pakistan
[2]Department of Plant Breeding and Genetics, University of Agriculture, Faisalabad, Pakistan
[3]State Key Laboratory of Cotton Biology, Institute of Cotton Research Chinese Academy of Agricultural Science, Anyang, Henan, China
[4]Agricultural Research Service, Southern Plains Agricultural Research Center, College Station, United States Department of Agriculture, USA
[5]Institute of Molecular Biology and Biotechnology, Bahauddin Zakariya University, Multan, Pakistan
[6]School of Agriculture Sciences, Zhengzhou University, Zhengzhou, China

Received: 18.12.2020 • **Accepted/Published Online:** 12.04.2021 • **Final Version:** 00.00.2021

Abstract: Cotton is an essential fiber producing crop in the world. It also supports additional industries by providing high quality oil and protein in the form of cottonseed cake. Currently, there is an urgent need to increase lint yield, fiber quality, and resistance to biotic and abiotic stresses due to rising pressure from a global population and possible supply shortages from the effects of erratic climate changes. Classic plant breeding and transgenic strategies need more genetic breakthroughs to support the increasing pressure for fiber quantity and quality. A potential for rapid increases in crop improvement is in various state-of-the-art gene editing technologies. Genetic research in simple micro-organisms revealed novel enzymes involved in natural sequence editing in cells, and they were successfully applied to gene editing in model plants through a system called clustered regularly interspaced short palindromic repeats-Cas9 (CRISPR-Cas9). This and other enzymatic systems are heralded as providing numerous possibilities for creating genetic variation for crop breeders. However, gene editing in agriculture is most effective when focused on achieving transmissible changes by inducing targeted mutations in genes involved in yield or quality attributes. The newly emerged CRISPR-Cas tools should accelerate future research in cotton breeding because they can be utilized efficiently for gene editing without the need for foreign gene insertion. Gene editing with CRISPR-Cas is achieved through the modification of gene regulatory mechanisms, enzymatic activities, and epigenetic factors as well as insect/pest gene drive technology, RNA targeting, and, more recently, single base and prime editing.

Key words: CRISPR-Cas variants, genome engineering, protein nutrition, sustainable cotton production, vegetable oil

1. Introduction

Deoxyribonucleic acid (DNA) was established as a functional component of life hereditary material by 1944, and its chemical and physical attributes were discovered by Watson and Crick, which paved the way for the breakthrough that revealed the structure of this molecule (Watson and Crick, 1953). The double helix revolutionized biology and other allied disciplines because much of the form and function of living beings and their inheritance in progeny could be attributed to chromosomes and individual DNA sequences revealed by the DNA structure. Since this discovery, plant breeders and biotechnologists have rigorously mined the possible strategies to tailor DNA for enhanced viability and improved performance. Before the identification of DNA, proteins were long thought to be the sole molecules responsible for gene expression and inheritance. After this discovery, critical links were established between DNA and enzymes. Enormous scientific breakthroughs were made in DNA enzymology during the last century. However, the discovery of restriction and other enzymes that enable a 'cut and paste' of DNA sequences opened a new era of genome tailoring (Smith and Welcox, 1970; Loenen et al., 2013). The ability to use proteins to reverse engineer DNA was another major advancement in genetics.

Modern agricultural practices have made significant strides in achieving higher crop production. Commercially grown crop plants are being produced with altered genetic information aiming to improve yield and quality of the product (Khare and Chauhan, 2020). Such advances in genetic engineering have provided a progressive tool for improving production potential especially when conventional plant breeding has encountered genetic bottlenecks and loss of original sources of genetic

* Correspondence: tehseenazhar@gmail.com

variation. Plant biologists are using this information to increase crop yields and resilience and are reshaping the genetic engineering landscape with more precision. Conventional plant hybridization encounters barriers when crossing different species, increased generations to overcome linkage drag, and increased risks of disturbing elite pedigrees. Therefore, an efficient method was needed for site-directed modifications in plant genomes to alter specific genes in elite germplasm, without wholesale genome changes. These new locus-specific techniques are exciting methods to dissect plant DNA and engineer crop plants that can meet the growing need for food, fiber, and fuel worldwide.

Urbanization along with a growing human population and the increasing threats of climate change will complicate the issues of global food security (Ruel et al., 2017). Technological advances and bringing new land under cultivation has increased crop yield and efficiency. However, this intensification is not adequate to meet future demands for a sustainable agriculture system (Laio et al., 2016). Plant biotechnologists are increasingly employing genetic engineering to improve crop varieties. Researchers first employed genetic engineering when they utilized enzymes to cut and paste DNA sequences and put them in biological vectors (*Agrobacterium*) to develop the first antibiotic resistant transgenic tobacco (Bevan et al., 1983). This technique spawned decades of genetically modified (GM) crop development and now more than 190 million hectares of land are under GM crop cultivation worldwide (Mandal et al., 2020).

The long history of engineering crops for higher yield, better nutritional quality, and stress resistance combined with the recent sequencing of genomes of various plant species have made biotechnology a viable option for the introduction of other desirable, novel traits (Peng et al., 2020). In the past, plant scientists used mutagens to generate genetic variation, but this method lacked precision to target a specific DNA sequence and, therefore, resulted in a very low percentage of usable, viable mutant plants. Targeting specific genomic locations is a direct and rapid way to edit, delete, or add genomic sequences with more precision. Site-specific interactions between proteins and DNA sequences also play roles in gene expression and modifications (Ren et al., 2000). The discovery of enzymes that repair or modify sequences has opened the possibility of using the existing genome instead of introducing a new foreign sequence of DNA. The sites of these sequences in the genome are also critical to their expression and function. A means to modify them in situ is preferable to the insertion of new sequences via Agrobacterium or other methods into a random location in the genome. Therefore, the ability to engineer such proteins would reveal numerous practical applications by targeting any desired DNA sequences.

2. Genome editing technologies

The scope of gene editing technologies and their potential applications both in agricultural and health sciences has risen enormously in the last few years (Doudna, 2015). In general, its wide-ranging utility can identify and modify selected DNA sequences. However, the application of gene editing to create new plant varieties, particularly in field crops like cotton, is a challenging area (Mao et al., 2019). Our limited understanding of stress biology in cotton along with complexities associated with ploidy level and their recalcitrant nature can pose significant hurdles when trying to upscale functional genomics to plant trait development (Aslam et al., 2020). A wide range of research on genes and their function in related plants or model crops have identified candidate genes that might improve yield or other traits in cotton. Once target genes are identified, the real challenge is using enzymes to modify their sequence. Enzymes are needed to cut and unravel the sequence so that it can be deleted, replaced, or rewritten with an altered sequence. In order to catalyze the double stranded break (DSB) at a site-specific genomic location, engineered nuclease enzymes are being used to induce the selected DNA modifications at or near the cut site (Curtin et al., 2012). In fact, there are two natural pathways by which a DSB can be repaired: (i) error prone nonhomologous end joining (NHEJ) and (ii) highly efficient homologous directed recombination (HDR). To induce predefined modifications at specific genomic sites, HDR can be exploited (Puchta et al., 1996). NHEJ may result in knocking-out genes (Kirik et al., 2000) and insertion/deletion of DNA sequences anywhere in a genomic region rather than at a specific site (Siebert and Puchta, 2002).

There are several naturally occurring enzymes with the known property of inducing DSB in DNA strands, but these enzymes are limited by certain specificities or requirements (Takeuchi et al., 2011). Scientists have continued to search for enzymes that modify DNA and studied their sequence specificities and requirements to enable lab scale engineering and manipulation of designer enzymes. As they are targeting functional sequences and not general restriction sites, designer enzymes must have an intrinsic ability to recognize a long and specific DNA sequence (Lee et al., 2016).

While studying oocytes from *Xenopus laevis*, Miller et al. (1985) discovered a repeating protein motif with a zinc centered domain having repeating cytidine and histidine residues. Proteins with this motif became known as zinc finger proteins (ZFPs), and their discovery began the journey to in vivo editing (Klug and Rhodes, 1987). A significant development was the introduction of zinc finger nucleases (ZFNs) based fusion of ZFPs with the type II-S restriction enzyme FokI (Smith et al., 2000). The

efficacy of ZFNs for inducing targeted DNA modifications was initially demonstrated in *Drosophila melanogaster* and *Homo sapiens* (Bibikova et al., 2003). The preliminary reports of using ZFNs mediated plant genome engineering were described in model plants, *i.e*., *Arabidopsis* and tobacco (Lloyd et al., 2005; Wright et al., 2005). So far, ZFNs based targeted gene mutation and correction via induction of DSB in targeted DNA has shown promising results in different crop plants, *i.e.,* maize and soybean (Shukla et al., 2009; Ainley et al., 2013; Petolino, 2015).

Similar to ZFPs, other specific DNA binding proteins have been identified in the plant pathogenic bacteria *Xanthomonas*. This bacterial pathogen is known for its devastating ability to infect a wide range of plants including tomato, citrus, rice, and soybean (Kay and Bonas, 2009; Boch and Bonas, 2010). During infection, *Xanthomonas* injects the effector proteins known as transcriptional activator-like effectors (TALEs) into the cytoplasm of plant cells. These transcription activators alter the host's gene expression by binding with specific promoter sites and efficiently reproduce host transcription factors (Kay and Bonas, 2009).

Genome engineers have successfully decoded the DNA recognition mechanism of TALEs, which has provided an alternative platform for the wide-ranging application of emerging biotechnology tools (Bogdanove et al., 2010; Khan et al., 2017). The fusion of TALEs with FokI (a process known as TALEN) has made it possible to create targeted DSBs at specific DNA sequences (Christian et al., 2010). Until now, TALEN technology has been used to create targeted genome modifications in various model plants, such as *Arabidopsis*, tobacco, maize, wheat, tomato, and potato (Cermak et al., 2011; Mahfouz et al., 2011; Li et al., 2012).

Because it is common in nature for infectious organisms to use enzymes to manipulate host DNA, scientists have studied their mechanisms and, conversely, the host cell mechanisms used to alter or repair this damage. Bacteria and archaea are abundantly diverse and the most ubiquitous living organisms of the universe. Most of our understanding of antiviral immunity in bacteria has been focused on abortive phage phenotypes, restriction modification systems, innate defense systems, toxins, and antitoxins (Stern et al., 2010). Immune systems of host cells are designed to recognize and act upon foreign molecules and organisms. Nucleic acids of the invader are sometimes used as excellent locators of the foreign organism or to even correct the damage. With a known sequence of the infectious organism, the host cell enzymes can seek out and target the foreign sequences. In the last few years, technological advances have led to the discovery of a mechanism that appears to function in this manner. This mechanism is known as the clustered regularly interspaced short palindromic repeats-CRISPR associated (CRISPR-Cas) system. With a CRISPR-Cas like immune system in prokaryotes, RNA guided cleavage is carried out to target and eliminate the genetic parasites through base pairing with a specific nucleotide sequence (Makarova et al., 2011).

3. The rise of the CRISPR-Cas9 system

The rapid evolution of gene editing technologies originated with the pioneers who identified the CRISPR system and their extraordinary insight and ability to decode microbial repeats, identify characteristics of adaptive immune systems, characterize their biological meaning, and subsequently remodel the system for genome engineering. In 1989 at the University of Alicante on Spain's Costa Blanca, Francisco Mojica, a doctoral student, was working on *Haloferax mediterranei*, an archaeal microbe with extreme salt tolerance. He found a nearly perfect and palindromic repeated 30 nucleotide base sequence, separated by a roughly 36 base spacer sequence that did not overlap with any known repeats in microbes (Mojica et al., 1993). He discovered a similar nucleotide repeat in *H. volcanii*, which closely resembled the structure in *E. coli* reported by Ishino et al., (1987). He quickly proposed the existence of similar repeats in distant microbial species and reported the new class of nucleotide repeats called short regularly spaced repeats (SRSRs) in prokaryotes (Mojica et al., 1995). Later, at his suggestion, the name of the repeats was changed to CRISPRs (Mojica and Garrett, 2012). CRISPR loci have been identified in more than 15 microbes, including *Clostridium difficile* and *Mycobacterium tuberculosis* (Mojica et al., 2000). Other research groups have compiled key functional characteristics of CRISPR loci in the vicinity of specific CRISPR-associated (Cas) genes (Jansen et al., 2000).

One of the spacers of the CRISPR locus in the *E. coli* strain was sequenced by Mojica in 2003, and it matched the P1 phage sequence that infected several other strains of *E. coli*. Moreover, the strain containing the spacer sequence was recognized to be resistant to P1 infection. Shortly thereafter, he theorized that the data for an adaptive immune system that can protect microbes against particular infections must be translated by CRISPR loci. After a series of rejections of Mojica's manuscript from various journals namely, Molecular Microbiology, Nature, Nucleic Acid Research Journal and PNAS, finally Molecular Evolution published his article describing the function of CRISPR (Mojica et al., 2005). Two similar reports were published in Microbiology by independent researchers who proposed, 1) that CRISPR loci might represent a memory of past genetic hostilities, and 2) loci could be operated by anti-sense RNA inhibition of phage gene expression, respectively (Bolotin et al., 2005).

Among various Cas gene products, Cas9 is the unique protein that has the RuvC- and HNH nuclease domains which are required for interference (Jinek et al., 2014). During the last two decades, Marraffini and Sontheimer discerned that the Cas9 product was a restriction enzyme and demonstrated its potential to carry out DNA cleavage in an in vitro study. They were the first to predict that the CRISPR system could be used for genome engineering (Marraffini and Sontheimer, 2008). They also attempted to file a patent claim demonstrating the use of CRISPR to cut or correct DNA sequences in eukaryotic cells, but unsatisfactory experimental validation prevented acceptance of this initial patent claim. A contentious battle between Feng Zhang of the Broad Institute and Jennifer Doudna of the University of California, Berkley occurred over the intellectual-property rights to the potentially lucrative CRISPR-Cas9 technology. The US Patent and Trademark Office (USPTO) awarded the first patent for CRISPR-Cas9 mediated gene editing technology to the Broad Institute of MIT and Harvard (Ledford, 2017).

Since the realization of CRISPR as a programmable restriction enzyme (Marraffini and Sontheimer, 2008), several scientists have pursued CRISPR technologies as a commanding option for site specific genome engineering. By mid-2012, Feng Zhang at the Broad Institute first reported a breakthrough assembly system composed of tracrRNA, a Cas9 endonuclease from *S. pyogenes* or *S. thermophilus*, and a CRISPR array. According to Zhang, it was possible to mutate genes by targeting 16 sites in human and mouse genomes, and they observed a high efficiency and accuracy of deletions via the NHEJ repair mechanism and insertion of new sequences via HR with a repair template. By 2012, news of successful in vivo genome editing was presented, while other research groups were racing to conduct key validation experiments, which indicated that a genome cleavage was not editing. Another group used the CRISPR tool to demonstrate low level cutting at one genomic site (Jinek et al., 2012). The many advantages offered by CRISPR in genetic engineering have attracted researchers from all avenues and recently resulted in a 2020 Nobel Prize in Chemistry.

3.1. Genome editing of cotton with the CRISPR-Cas9 system

Cotton is the major fiber crop and a valuable source of oil and protein (Peng et al., 2020). Despite a shift towards the use of synthetic fibers in a range of applications, cotton is still the most important natural fiber around the globe (Campbell et al., 2018) and desirable as a renewable, 'green' source of fabric and clothing. Although conventional breeding and transgenic technologies have shown their potential in the development of improved cotton cultivars, it still takes years to produce new GM varieties. The developments in genome sequencing and gene editing technologies show promise to decrease the time needed to develop new varieties and enhance sustainable cotton production (Peng et al., 2020). Recently, the CRISPR-Cas9 system has been engineered into a powerful genome editing tool. The CRISPR-Cas9 based gene editing system has gained much attention from both the academic and industrial sector, and it is being researched by several laboratories to edit candidate cotton gene(s). The CRISPR-Cas9 system could transform next-generation gene editing because it is an inexpensive and efficient way of inducing site-specific genetic modifications, regulation of gene expression, and epigenetic regulations (Figure) (Doudna and Charpentier, 2014; Shen et al., 2017). It allows for desirable genetic modifications in plants, and it is being widely pursued as an alternative to lengthy and expensive classical breeding and transgenic approaches (Figure). It may be the ideal precursor to plant germplasm development because it can change specific genes in elite pedigrees without the need to recombine them in lengthy breeding schemes. It also allows for the genome to still be classified as conventional and not subject to the rigorous testing and licensing required with transgenic germplasm. Moreover, there is no prerequisite of engineering a Cas9 protein for an active CRISPR-Cas9 cassette for screening of multiple gRNAs for each target gene (Mubarik et al., 2016; Wang et al., 2018a). Early reports of CRISPR-Cas9 in model plants have demonstrated the practical application of CRISPR-Cas9 as a genome editing tool for a variety of crop plants like *N. benthamiana* (Nekrasov et al., 2013) and Arabidopsis (Jiang et al., 2013).

The technology of CRISPR-Cas9 has provided a strong incentive for researchers to work on these powerful tools for improvement in cotton research. Here, we highlight the potential applications of a CRISPR-Cas9 system to improve lint yield, quality, and tolerance to biotic and abiotic stresses. During 2017, the first report of CRISPR-Cas9 mediated gene editing in cotton was described by Janga et al., (2017), where the gene editing tool was used in targeted knock-out of an already integrated GFP gene in the cotton genome. Another research group reported the parallel editing of three homoeologous genes (*GhPDS*, *GhCLA1*, and *GhEF1*) in the cotton genome (Gao et al., 2017). A number of cotton genes have subsequently been targeted using the CRISPR-Cas9 system, including vacuolar H+-pyrophosphatase (*GhVP*) (Chen et al., 2017), discosoma red fluorescent protein2 (*DsRed2*) (Wang et al., 2018b), nucleotide-binding (NB)-ARC domain-containing disease resistance protein (ARC), MYB44 transcription factor (*MYB44*), and AP2/B3-like transcription factor (*AP2*) (Li et al., 2019).

For cotton improvement, a priority is the study of fiber initiation and development at the cellular level. With high efficiency and no off-targeting, Li et al. (2017) knocked-

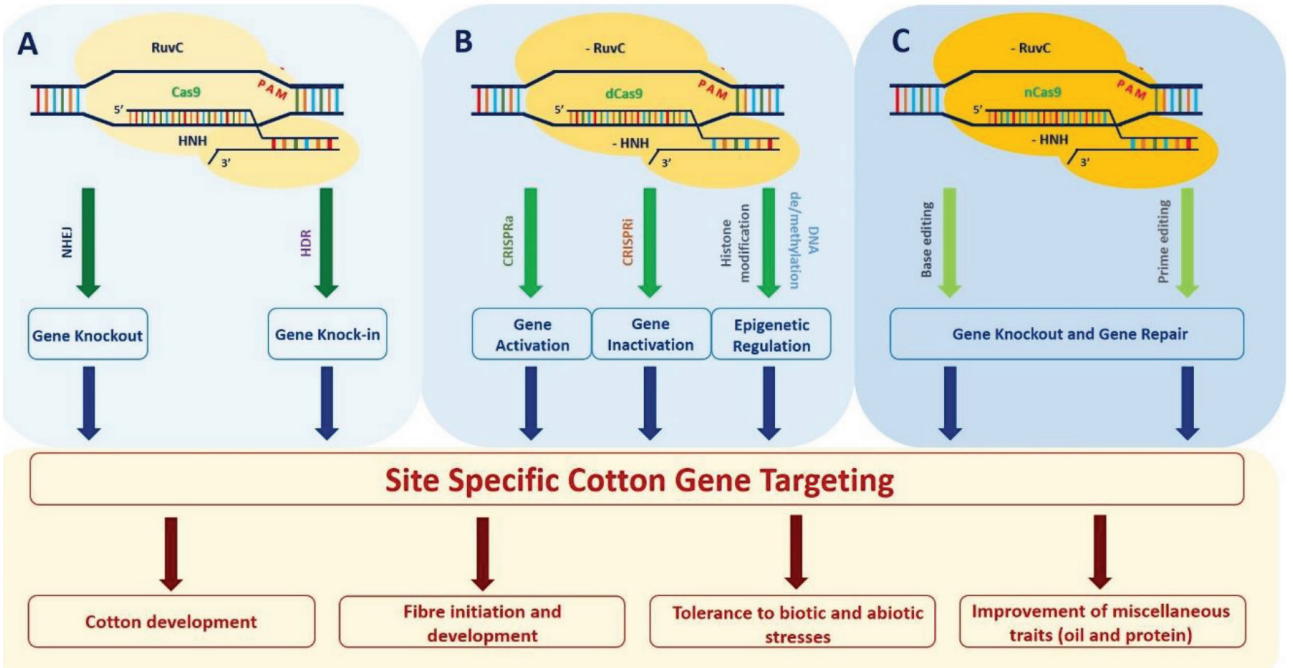

Figure. Applications of CRISPR-Cas9, dCas9, base editing, and prime editing technologies in cotton breeding programs. (a) Site specific knock-out and knock-in of cotton genes to improve elite cultivars. (b) Genetic and epigenetic gene regulation by using catalytically dead Cas9 (dCas9) fused with transcription activator, repressor, and epigenetic regulators. (c) Targeted and pre-defined single base modifications with nickase Cas9 (nCas9) fused with APOBEC (apolipoprotein B editing catalytic polypeptide) and reverse transcriptase enzymes.

out a *MYB-25* like transcription factor gene in cotton. Without modifying other phenotypic characteristics, the knock-out line exhibited a fiber-less plant phenotype (Li et al., 2017). In another study, *GhALARP* (a gene encoding an alanine-rich protein pre-dominantly expressed in cotton fibers) was mutated using the CRISPR-Cas9 system. Such findings provided the resources to further studying the role of *GhALARP* and related genes in the development of cotton fiber (Zhu et al., 2018) (Table 1). Although, cotton is grown for its lint, approximately 1.6 times more seed by weight is also produced. Cottonseed contains about 23 percent protein in addition to oil. Globally cottonseed is providing more than 10 million metric tons of protein and can potentially fulfill the dietary requirements of approximately 550 million individuals (Rathore et al., 2020). Unfortunately, due to the presence of toxic gossypol compounds in the seed, this abundant resource of vegetative protein cannot be used for food or even as feed for monogastric animals. Because the *MYB-25* transcription factor was successfully knocked-out, the CRISPR-Cas9 can be used in combination with a seed-specific promoter to remove gossypol glands from cottonseed and make it possible for human and animal consumption (Janga et al., 2019).

Enhancing resistance/tolerance to biotic and abiotic stresses is another important application of genome editing tools. *Verticillium* wilt in cotton, known as "Cotton Cancer", is a devastating disease causing an economic loss of more than 250 million US dollars annually in China (Wang et al., 2016). The *Gh14-3-3d* gene in cotton was mutated using the CRISPR-Cas9 system, and homozygous mutated plants without the vector backbone exhibited resistance to *Verticillium dahlia* when compared with the wild-type (Zhang et al., 2018) (Table 1). Therefore, such mutants could be used directly as a potential source to breed resistant cultivars and can sidestep the time-consuming and costly procedures required to evaluate the safety of transgenic plants derived by other means. Whitefly transmitted cotton leaf curl disease (CLCuD) is also a major threat to cotton production in Pakistan, India, and other parts of Asia (Mansoor et al., 2003). In several studies, the expression of single and multiple gRNAs targeting cotton leaf curl virus DNA has demonstrated effective control of CLCuD (Mubarik et al., 2019; Yin et al., 2019).

Drought is a major factor that negatively affects the growth, lint yield, and quality of cotton, and reduced water availability is expected to worsen with an increased population and climate change. In plants, the roots serve as the key indicator organ for abiotic stress signaling and response. Therefore, improving lateral root formation could increase root surface area, effectively improve

cotton growth, and potentially enhance lint yields particularly under drought conditions. Earlier studies have shown that substantial over-expression of the rice arginase gene (*OsARG*) in upland cotton inhibited the development of lateral roots (Meng et al., 2015). Recently, the *GhARG* gene in cotton was knocked-out on both the A- and D-chromosomes using the CRISPR-Cas9 system. This CRISPR induced knock-out mutant has exhibited significant development of the lateral root system, increased lint yield, enhanced nutrient absorption, and improved adaptability to water limited and high saline soils (Wang et al., 2017) (Table 1).

In polyploid crops, CRISPR-Cas9 based gene editing can be used with high efficiency to manipulate multicopy genes. In addition to identifying specific genes that enhance traits such as fiber length and strength, biotic and abiotic stress tolerance are still a major constraint to cotton production. Even though whole genome sequencing and multi-omics approaches have been carried out and genes associated with these characteristics have been identified, their complete functions remain unknown. Advances in genome sequencing and omics technologies in tandem with CRISPR-Cas9 based gene editing can be incorporated to identify useful genes including those associated with initiation and growth of cotton fiber and resistance to environmental stresses. Candidate genes can be knocked-out, altered, or upregulated to confirm, quantify, or rule out their role in cotton yield, quality, or other economically important attributes. However, high editing efficiency is still based on CRISPR-Cas9 components being delivered to rigid plant cells like cotton (Sandhya et al., 2020).

3.2. Base editing: a new way to alter DNA

CRISPR-Cas9 mediated mutagenesis holds great promise in developing improved cotton cultivars to meet increasing fiber and food demands. Specifically, CRISPR-Cas9 mediated single base editing could produce elite trait variants that help to accelerate crop improvement programs. The recent developments in CRISPR-Cas9 using base editors have enabled efficient and precise base conversions in crop plants (Kang et al., 2018). The use of base editors is an exciting addition to the CRISPR system by further improving its efficacy in plant genome editing. Early studies in Nature Biotechnology showcase the quick advance of the technology and its potential applications in plants like tomato and rice (Shimatani et al., 2017). Functional genomics has been rapidly facilitated by recent developments in cotton genome sequencing, but current success in cotton remains far behind the range of achievements obtained with model plants.

As already outlined, typical CRISPR-Cas9 induces DSBs that stimulate endogenous repair mechanisms either by error-prone NHEJ or highly efficient HDR repair. Since it is an allotetraploid, several alleles in the cotton genome are similar with few SNPs, and, therefore, the typical CRISPR-Cas9 system is futile as the functional study of homologous alleles involves a single nucleotide polymorphism (Mishra et al., 2019). Recently, two cotton genes (*i.e. GhCLA* and *GhPEBP*) were targeted for their obvious phenotype without observable off-target effects. A robust cytosine base editor system consisting of a cytidine deaminase domain fused with nicked Cas9 (nCas9) exhibited a high-base editing efficiency (Qin et al., 2020) (Table 1).

This novel genome editing approach combines the idea drawn from chemical biology and genome engineering to allow the site-specific direct chemical substitution of one target base into another without stimulating DSB (Nishida et al., 2016) (Figure). It is critically important to be able to make single base modifications because most diseases are associated with point mutations from random conversion of C-G to T-A base pairs (Gaudelli et al. 2017). Moreover, all four base transition mutations can be generated by combining adenine and cytidine base editors. It is therefore an efficient and robust method for directed base editing and will provide significant technological support for functional genome analysis, crop genetic improvement, and breeding of new cotton varieties. However, existing base editing technologies can execute substitution mutations only, allowing modifications of C-G to T-A and cannot introduce deletions, insertions, and transversions (Gaudelli et al., 2017).

A new genome editing technique called "prime editing" can integrate indels and base-to-base conversions with fewer inadvertent products at the targeted site (Van-Eck, 2020) (Figure). Recently, prime editing was applied in wheat, rice, maize, and potato (Jiang et al., 2020; Lin et al., 2020). Prime editing in plants is a very new approach, but this technology holds immense potential for diverse plant gene editing applications. Prime editing has wide flexibility to accomplish various forms of edits in plant genomes. It has a significant potential to develop superior cotton cultivars that provide increased lint yield, quality, resistance to various biotic and abiotic stresses.

4. Cotton genome editing beyond CRISPR-Cas9

The CRISPR-Cas9 system provides a versatile tool for plant gene editing both in model and crop plants. In addition, nongenetically modified (nGM) crop plants have also been produced by CRISPR-Cas9 mediated gene editing (Kanchiswamy et al., 2015). In the United States, nGM crop plants have been approved for commercial production (Waltz, 2016). CRISPR–Cas9 has some limitations, such as restricted target of sequences due to the requirement for a protospacer adjacent motif (PAM), the large size of the Cas9 protein poses difficulties to deliver into cells and off-target effects. Researchers continue to identify and

characterize new enzymes that have different sequence requirements so that they can expand the number of sequences available for modification.

CRISPR-Cpf1/Cas12a, a recently discovered class II type V endonuclease system, has novel and superior features that lack the Cas9 from *S. pyogenes* (SpCas9) (Zetsche et al., 2015) (Table 2). SpCas9 produces blunt ends in a DNA sequence, while Cpf1 generates single strand ends of four or five nucleotides. The targeted DNA molecule is cleaved by Cpf1 with a crRNA shorter than the gRNA for SpCas9 (43nt versus 100nt). It can be used for multiplex genome editing with a tandemly arrayed pre-crRNA expressing gene cassette that transcribes multiple crRNAs processed by Cpf1 and contains RNaseIII activity for pre-crRNA processing. Recent reports show that CRISPR-Cpf1 exhibits nonsignificant to no off-target activities (Tang et al., 2017; Xu et al., 2017). Among several proteins in the Cpf1 family, LbCpf1, AsCpf1, and FnCpf1 are commonly used in genome editing experiments (Tak et al., 2017). The usefulness of CRISPR/Cpf1 has initially been demonstrated for targeted mutagenesis in Arabidopsis and rice (Endo et al., 2016).

Recently, a LbCpf1 plant expression vector containing 23-nt crRNA has been used to target the cloroplastos alterados (*GhCLA*) gene in allotetraploid cotton. The results indicated more than 80% editing efficiency and no off-target effects. These findings are equivalent to previously reported base editing in maize and rice (Tang et al., 2017; Zong et al., 2017). The edited phenotypes were stably transferred into subsequent generations and some homozygous mutants also obtained in T_1 generation (Li et al., 2019). In another study, the pigment gland formation (*PGF*) gene was silenced using the CRISPR-Cpf1 system under different temperatures in cotton. The results indicated that maximum temperature for active CRISPR-LbCpf1 in cotton was 34 °C. As a result of base

Table 1. Applications of CRISPR-Cas systems in cotton (*Gossypium* spp.) improvement.

Gene editing method	Targeted gene	Type of genetic modification	Study objective	Reference
CRISPR-Cas9	*GFP*	Gene disruption	Loss of function mutation	Janga et al. 2017
CRISPR-Cas9	*GhPDS, GhEF1, GhCLA1*	Gene disruption	Loss of function mutation	Gao et al. 2017
CRISPR-Cas9	*GhCLA1, GhVP*	Gene disruption	Targeted gene editing in protoplast	Chen et al. 2017
CRISPR-Cas9	*GhMYB25-A, GhMYB25-D*	Gene disruption	Targeted gene editing of fibre related genes	Li et al. 2017
CRISPR-Cas9	*GhARG*	Gene disruption	Improve lateral root formation	Wang et al. 2017
CRISPR-Cas9	*dsRed2*	Gene disruption	Loss of function mutation	Wang et al. 2018
CRISPR-Cas9	*AP2, MYB44, ARC*	Gene disruption	Study off-target activity of CRISPR-Cas9	Li et al. 2018
CRISPR-Cas9	*GhALARP-A, GhALARP-D*	Gene disruption	Editing of gene expressed in cotton fibre	Zhu et al. 2018
CRISPR-Cas9	*Gh14-3-3D*	Gene disruption	Resistance against *Verticillium dahliae*	Zhang et al. 2018
CRISPR-nCas9-APOBEC	*GhCLA, GhPEBP*	Base editing	Test the efficiency of base editing in cotton	Qin et al. 2020
CRISPR-Cpf1	*GhCLA1*	Gene disruption	Targeted gene mutation	Li et al. 2019
CRISPR-Cpf1	*GhPGF*	Gene disruption	Gossypol free cotton	Li et al. 2020

Table 2. CRISPR toolbox for plant gene editing.

CRISPR-Cas system	Type	Core components	Functions	Reference
CRISPR-Cas9	Type II	Cas9, sgRNA	DNA targeting with Cas9 and sgRNA	Jinek et al., 2012
		dCas9, sgRNA	Epigenetic modifications, transcriptional regulation, DNA or RNA tracking	Qi et al., 2013
CRISPR-Cpf1	Type V	Cpf1, crRNA	DNA targeting with Cpf1 and crRNA	Zetsche et al., 2015
CRISPR-C2c2	Type VI	C2c2, crRNA	RNA targeting with C2c2 and crRNA	Abudayyeh et al., 2016
		dC2c2, crRNA	RNA tracking, transcriptional regulation	Abudayyeh et al., 2016

editing, a homozygous gossypol-free nontransgenic line was identified that could be used as a new germplasm for cotton breeding programs (Li et al., 2020) (Table 1). It is foreseen that a highly precise and effective CRISPR-Cpf1 mediated plant gene editing system will provide an alternative to the CRISPR-Cas9 based gene editing system in cotton.

In addition to Cpf1, ~53 other candidates for a CRISPR-Cas class II endonuclease were identified. Among them, Cas13a (previously known as C2c2) has a unique property of targeting single stranded RNA (Table 2). This provides an opportunity to induce gene knockdown by targeting mRNAs (Seletsky et al., 2016; Burstein et al. 2017). Still, it is in the nascent phase with limited reports in plants (Chaudhary, 2018; Khan et al., 2018; Zhang et al., 2019) and remains to be applied in cotton. Cas13a also serves a dual nuclease activity, like Cpf1, and catalytically inactive Cas13a also maintains targeted RNA binding activity that can be used for programmable tracking of transcripts in live cells (Abudayyeh et al., 2017).

5. CRISPR based gene drive and cotton pest management

In cotton fields, more than 1300 types of plant feeding pests, including insects and mites have been reported (Tarazi et al., 2019). The most damaging pests include whitefly, cotton bollworms, thrips, dusky cotton bug, aphid, jassid, and termites. In addition, losses in cotton can also occur due to high population pressure from whitefly and their transmitted begomoviruses (Sattar et al., 2013). Currently, insect-pest control in cotton fields depends largely on conventional pesticides. However, extensive application of pesticides has led to serious ecological problems, including hazards to human and animal health, development of resistance in target pests, and environmental pollution (Sharma et al., 2020). The reliance on pesticides comes at a price in that it harms natural predators and other nontarget species such as pollinators.

Transgenic Bt cotton is often used to manage numerous lepidopteran and coleopteran destructive insect pest species in most production areas of the world. This reduces application of pesticides, and toxins are located only in host plant cells and delivered only to feeding insect pests (McLaughlin and Dearden, 2019). Because the extensive use of Bt cotton has reduced the application of broad-spectrum insecticides, it may have triggered outbreaks of secondary pest species (Gowda et al., 2016). Limited use of insecticides in Bt cotton will continue to increase the population of sucking pests, which often vector diseases, often a more serious component of the insect-pest complex in cotton (Men et al., 2005).

CRISPR-Cas9 based gene editing can create opportunities to control pest species and/or intervening transmission of pathogens by them (Mubarik et al., 2020). When used properly, gene editing of pest species is transmissible through sexual reproduction, and the target gene(s) is spread across a target population (Pixley et al., 2019). In the past decade, use of gene drive technology-based approaches has been proposed to control various invasive insect species (Deredec et al., 2008; Hodgins et al., 2009), but application has been limited to mosquitoes (Alphey, 2013). However, the CRISPR-Cas9 system has been given consideration to control invasive species (Esvelt et al., 2014). This novel approach has been realized as a breakthrough with the ability to perform largescale replacement or eradication of a target gene or genes. In agriculture, some potential gene drive applications are underway. One such example is to control citrus greening, a devastating bacterial disease (*Candidatus liberibacter*) vectored by the Asian citrus psyllid (*Diaphorina citri*) and other psyllid species (da-Graca et al., 2016). Another example is to control spotted wing *Drosophila* (*Drosophila suzukii*), an invasive fruit fly from eastern Asia, which causes extensive damage to ripening berry and stone fruits and markedly increases pest management costs (Asplen et al., 2015).

Gene drive technology has many potential applications against insect pests to improve agriculture production. Compared to other pest control interventions, gene drive technology appears to be more cost effective, precise, and distinctively less controversial (Courtier-Orgogozo et al., 2017; Eckhoff et al., 2017). Gene drive-mediated pest control is enticing for agricultural entrepreneurs because it provides an opportunity to alter gene flow in a pest species and achieve more directed and lasting control in contrast to traditional host plant GMO technologies. It is expected that this technology can eradicate pests once the first gene edited organisms are introduced into the crop landscape. It is well documented that genes for key physiological and metabolic functions in plants can be manipulated with a CRISPR-Cas9 based gene drive cassette (Rostami, 2020). Conversely, it can also be engineered to knock-out any gene at any chosen site within the genome. The reproductive success of a population is key to its survival, and, theoretically, if gene drive removes a male or female specific gene necessary for reproduction, it can lead to extinction of harmful insect species (Burt, 2003). Indirectly impacting species reproduction by targeting physiology or metabolism is less effective because of genetic variability and other mechanisms species have to escape selection pressures. Species eradication by targeting genes involved directly in reproduction appears to be the best hope of reducing or eliminating populations. When used properly, gene drive technology is used only to target the reproductive success of a pest species, and will be of less risk because it is not likely to encourage selection for genes of resistance to other mechanisms of control, like insecticides or GM host plant toxins.

In summary, gene drive experts have developed a deeper understanding of host plant and pest interactions at a genetic

level, within and between species. Control methods have gone far beyond just applying toxins or engineering them within the crop plant. Now opportunities exist to modify existing genes within host plants for increased resistance, higher yield, and better quality. The technology is so effective that it can be used on the pest population to alter gene flow and population genetics, particularly with genes highly conserved and essential for reproductive success. Anyone working with this technology should have a comprehensive understanding of ecosystem dynamics and community ecology to explore ways to safely use new technologies to solve agricultural problems while reducing negative impacts on the environment. Given the potential significance of CRISPR-Cas9 based gene drive technology, these ecological traits should necessarily be modeled via their impact on individual populations and their life cycle, interactions with other species, as well as effects on other environmental elements. Could it also be used to control invasive weeds that cause yield losses, foster insect pests, host diseases, and require great expenditures in herbicide applications? Before gene drive experts rush for solutions to all these problems, they should keep in mind a holistic approach to move from uncertain risks and to focus on quantifiable hazards, which could turn out to be a challenging endeavor but one that consistently produces sustainable results.

6. Conclusion

The application of CRISPR-Cas9 and associated resources has erased many barriers to genome editing and has revitalized strategies of cotton improvement. However, these CRISPR resources need to be explored and established in cotton to fully realize their potential. Subsequently, engineering cotton with CRISPR-Cas system can help to reduce environmental stresses and disease attacks, which have an impact on overall cotton yield and lint quality. The CRISPR-Cas system now enables researchers to develop DNA-free editing in crop plants, which may remove the need for strict biosafety regulations as are required on traditionally developed transgenic plants. Furthermore, multiplex genome editing allows for the quick stacking of multiple traits in elite cotton germplasm, which has a significant effect on improving complex agronomic traits. Given the rapid increase of available cotton genomic information and improved plant transformation strategies in cotton, it is anticipated that recent advances in the CRISPR-Cas based gene editing system will bring a new generation of improved cotton cultivars to better meet increasing demands for quality fiber, oil, and protein.

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