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The ICAC Recorder

International Cotton Advisory Committee

SPECIAL ISSUE: CRISPR/CAS9 GENE EDITING IN COTTON

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The ICAC Recorder

Editorial

For most scientists, CRISPR (clustered regularly interspaced short palindromic repeats) is an exciting new gene editing tool. CRISPR is widely considered to have all the elements that can radically transform nature like never before. In the future, it might actually be recognised as the most important scientific discovery of the 21st century! As a result, the September 2019 edition of the ICAC RECORDER focusses exclusively on 'CRISPR for cotton improvement'.

But CRISPR is not just about a gene editing tool. It was actually discovered as God's own armoury gifted to tiny bacteria so that they could fight viral invasion. Science might never be more fascinating than this.

Picture this: A tiny little virus injects its DNA into a tiny bacterium so it could hijack the bacterial DNA and grow into a new virus. But the little bacterium swings into action! It understands the danger of the invading DNA, so it reads the DNA sequences of the virus and selects a short, 30-letter segment and copies it into its own genomic DNA to create a CRISPR library of the invading viral DNA pieces. The bacterium now is ready to combat viral attacks. As soon as a bacteriophage injects its DNA into the bacterium, the bacterium recollects its DNA spacer library to read and recognise identical sequences in the invading viral DNA to destroy it by cutting it into pieces. The bacterial CRISPR library keeps expanding with each invading new viral DNA, and that genetic information is passed on to its children for immunity against bacteriophages. One would have thought that the bacteria are smarter and that the bacteriophages would have gone extinct by now — but the bacteriophage viruses keep evolving too, in this God's game of chess. Numbering more than 10 quintillion (10^{31}), bacteriophages are likely the most populous living organisms on the planet.

Interestingly, the British microbiologist Ernest Hanbury Hankin, who worked in India, published a research paper on bacteriophages in 1896 in the journal *Annales de l'Institut Pasteur*. 10: 511–523. In it, he indicated the possible presence of bacteriophage viruses in the Ganges and Yamuna rivers that had the ability to control cholera due to bacterial infection. Twenty years later, the French-Canadian microbiologist introduced the term 'phage-therapy' to cure dysentery using bacteriophages. These scientists considered the bacteriophage viruses to be smarter than the bacteria. Almost 100 years later, scientists confirmed that not only were bacteria just as smart, they also had the CRISPR armoury — which is now used by scientists to edit genes in an absolutely precise manner to better understand gene functions, cure genetic disorders, and improve agricultural crops.

There is a raging debate whether CRISPR would be used for human welfare or if it could end up in the hands of a maverick scientist, who could distort the natural course of evolution in the pretext of creating 'better human beings' or 'elite organisms'. Charles Darwin's cousin, Sir Francis Galton, termed the coin 'eugenics' (well-born or good-genes) and suggested that the human race could be enhanced by retaining superior individuals and by eliminating the disabled and inferior. His idea created an era of havoc in human history across Britain, France, the United States and Germany, ultimately culminating in horrendous death camps and genocide.

A new chapter in the era of eugenics appears to have begun in 2018, when a Chinese scientist named He Jiankui claimed to have edited the genomes of twin girls by knocking out the *CCR5* gene, which would make them immune to the HIV virus. Jiankui's work not only drew flak from the scientific community, but also raised red flags against the potential misuse of the technology. It wouldn't be wrong to say that CRISPR technology is powerful enough to be used for human welfare — or misused for disastrous consequences, much like nuclear energy. The ICAC RECORDER does not touch upon those aspects of the debate, but would like agricultural scientists to consider all possible consequences before they attempt to modify natural evolution.

Information on CRISPR has grown by leaps and bounds over the past 20 years. There are brilliant reviews and videos on the internet that could provide general information to the reader on the potential of CRISPR, as well as the possible consequences.

There are four mini-reviews in this volume that describe CRISPR in the context of the extant gene editing technologies, biosafety regulatory mechanisms and the promise of gene editing for cotton improvement. I believe that the four mini-reviews in this volume of the ICAC RECORDER focus spotlight on the future of cotton through the prism of CRISPR/Cas. Happy reading.

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The ICAC Recorder

Mini-Review

CRISPR/Cas-based Genome Editing: A New Era for Transgenic and Precise Breeding in Cotton

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Abstract

It is a long-term dream for cotton scientists and researchers to breed new cotton cultivars with a high degree of precision. However, it requires about a decade to breed one new cultivar using traditional breeding technologies, which invariably present uncertainties due to a possible linkage drag of undesirable traits. Although transgenic technology brings huge benefits to cotton production, it can be used only for limited traits and the target genes are likely to get inserted at a random locus anywhere in the genome that may affect the function of other genes. The recently developed genome editing technologies, particularly CRISPR/Cas (clustered regularly interspaced short palindromic repeats, and the CRISPR associated protein Cas9) is opening a new era for transgenic development and precision breeding in cotton. This new technology allows breeders and scientists to edit any target genes and modify any cotton trait for agricultural purposes. CRISPR/Cas 9 has been used recently to modify cotton fibre and root development as well as respond to both biotic and abiotic stress. Many studies show that CRISPR/Cas is a powerful tool for precise breeding to significantly shorten the breeding time. It is widely believed that soon, elite cotton cultivars will be bred with high yield, quality and tolerance to environmental stress in an accelerated time using CRISPR/Cas and other related molecular tools.

Introduction

Cotton is one of the most important economic and fibre crops in the world, which has been cultivated in about a hundred countries across the globe for thousands of years. Since cotton was domesticated 5,000 years ago (Yoo and Wendel., 2014), man has been attempting to genetically improve cotton for higher yields, better quality, enhanced tolerance to different abiotic and biotic stresses, and also for better traits, such as early maturity suited for a short growing season. However, traditional breeding methods depend on a simple genetic cross and/or multiple crosses between two or more different elite germplasm lines; this is also generally followed by multiple self-crosses and/or backcrosses. This traditional breeding method is not only time-consuming, but also means 'breeding by chance' because we do not know the extent of genetic changes and where exactly which genetic information will be modified. Since the 1980s, transgenic technology has been used intensively to modify crops, thereby opening a new era for quickly breeding new cultivars with specific traits (Zhang, 2019). Transgenic technology significantly shortens the breeding time to modify an individual trait by inserting an individual exogenous gene into the plant genome. Cotton is amongst the first transgenic crops commercialised in the field. Insect resistant transgenic cotton crop, based on genes derived from the soil bacterium *Bacillus thuringiensis* (*Bt*) was first adopted by farmers in 1996. Subsequently, Bt cotton cultivation, has been widely adopted thereby resulting in huge economic and societal benefits in many countries, including the United States, China, India, Australia and Brazil (Zhang, 2019). However, currently, transgenic cotton in the field is limited to only two traits, such as insect resistant and herbicide resistant cotton. The major reason for the limited trait improvement is that the traditional transgenic technology only provides scope for overexpressing an individual gene by inserting it at a random location, which could many times interact with other genes or interrupt other gene functions. Moreover, it is hard to inhibit or knock out an individual gene with the current transgenic technology.

In the past decade, the development of genome-editing tools has revolutionised many fields, including improving crops. This also opens a new era for transgenic crops and precision breeding in cotton.

Genome editing provides significant opportunities for crop improvement

Genome editing is a special type of genetic engineering in which one molecular scissor cuts a DNA molecule at a specific site after which a double-strand break (DSB) is created at the desired location, which gets repaired by the internal cell molecular repair mechanisms, including homologous recombination (HR) and nonhomologous end-joining (NHEJ). Based on different repair mechanisms, the

repair will cause several different results, including nucleotide deletion and insertion thereby resulting in gene silencing (knockout). However, genome editing can also be used to insert an individual gene into a specific location of a chromosome or even be used to replace a non-functional gene with a functional gene and repair the gene function of a genetic mutant. Unlike the traditional transgenic technology, in which a desired gene is randomly inserted into a host genome, genome editing inserts a genetic material or deletes a short nucleotide sequence at a site-specific location. Thus, genome editing is also called precise genome editing.

The key for precise genome editing is to find a perfect molecular scissor that can efficiently cut an individual genome at a specific chromosome site. Since the first molecular scissor, 'meganuclease' was identified in the late 1980s and applied to edit an individual gene, genome editing has been quickly developed and applied to gene functional study and biotechnological breeding in both plants and animals (Wang *et al.*, 2016). Currently, there are four major types of nucleases that are used as molecular scissors:

- meganucleases,
- zinc finger nucleases (ZFNs),
- transcription activator-like effector-based nucleases (TALENs), and
- the clustered regularly interspaced short palindromic repeats and its associated protein commonly called Cas (CRISPR/Cas).

Meganucleases exist commonly in many microbial species and they recognise a large double-stranded DNA sequence of 12 to 40 base pairs (Silva *et al.*, 2011). Because they recognise a large DNA sequence, meganucleases have several advantages, including less off-target effects and thus less toxicity. However, because the construction of sequence-specific enzymes for a potential target DNA sequence is complicated, expensive and time consuming, meganuclease-based genome editing is being quickly replaced by other molecular scissors.

If meganucleases are the first generation of molecular scissors for genome editing, zinc finger nucleases (ZFNs) and transcription activator-like effector-based nucleases (TALENs) should be the second generation of molecular scissors. Both ZFNs and TALENs are specific DNA-binding proteins and have a similar mechanism to cut an individual DNA sequence.

ZFNs and TALENs have been successfully employed to knockout out several individual genes for studying gene function and even for altering important traits in crops (Wright *et al.*, 2014). However, due to the fact that constructing both ZFN and TALEN is time consuming and complicated, it has become tedious for many labs to perform the related research. The second generation of genome editing tools was quickly replaced by the third generation tools — the clustered regularly interspaced short palindromic repeats and its associated protein (CRISPR/Cas) system.

CRISPR/Cas is an RNA-guided DNA endonuclease enzyme associated with the CRISPR, it was firstly identified from a gram-positive bacterium *Streptococcus pyogenes*, and was found later in many bacteria and archaea (Sorek *et al.*, 2008). CRISPR/Cas system is used by bacteria to combat other DNA molecule invasion from bacteriophages or plasmid DNA (Sorek *et al.*, 2008). Thus, CRISPR/Cas is an adaptive immunity system employed by the bacteria. Since it was identified, scientists recognised that CRISPR/Cas could be developed as a powerful tool for editing plant and animal genomes. Currently, many Cas enzymes have been identified and modified for many purposes, including improving crop yield and quality as well as to combat different adverse environmental stresses.

CRISPR-based genome editing has shown huge promise for crop improvement (Sedeek *et al.*, 2019). First, CRISPR/Cas genome editing tools can be used to significantly improve crop yield. Improving crop yield is a long-term dream for scientists and breeders. However, due to the fact that crop yield is governed by quantitative traits and is controlled by multiple genes, the science of genetic improvement for yield enhancement becomes complex. Further, how the yield-associated genes regulate each other to control crop productivity is unclear as yet. Over the past two to three decades, scientists have tried their best to increase yields through transgenic methods, unsuccessfully though. Although a single major gene that controls yield has not been found as yet, many studies do show that a few genes negatively affect crop yield. It is hypothesised that yields could be improved by either inhibiting or silencing these negative genes. One recent study showed that CRISPR/Cas 9 genome knockout of genes *gn1a, dep1* and *gs3* — which are negative regulators of yields in rice — resulted in significantly enhancing yield parameters, including improved grain number, dense, erect panicles, and larger grain size, in rice (Li *et al.*, 2016). Another research using CRISPR/Cas 9 genome editing in rice also showed that knockout of grain weight negative regulators, *GW2, GW5* and *TGW6*, increased the grain size and weight (Xu *et al.*, 2016).

CRISPR/Cas genome editing also significantly increased plant resistance to various environmental abiotic and biotic stresses, including bacteria, fungal and/or virus-induced diseases. For example, CRISPR/Cas 9 edited Mildew-resistance Locus (mlo) gene mutants conferred heritable broad-spectrum resistance to powdery mildew in hexaploid bread wheat (Wang *et al.*, 2014). By targeting three different Potato virus Y PVR strains, Zhan and colleagues (2019) created potato genome-edited mutants with broad-spectrum resistance to multiple Potato virus Y (PVY) strains.

CRISPR/Cas: a new era for transgenics and precision breeding in cotton

Cotton is an allogeneic hexaploid, which makes it hard to obtain a pure line for trait enhancement through traditional breeding. Although *Bt* cotton and herbicide-resistant cotton have been successfully adopted by cotton farmers globally, transgenic breeding technology is still complex mainly because of the unpredictable genetic influences that may be caused by the insertion of the transgenes at random locations in the genome. Since the time CRISPR/Cas 9 technology was successfully deployed to study gene functions in plants in a precise manner, it has been attracting tremendous attention of the cotton scientific community. Currently, many research laboratories and breeders, including the biotechnology companies, have embarked into this exciting field by using CRISPR/Cas genome-editing tools to study gene functions as well as to improve cotton traits. CRISPR/Cas-based genome editing is thus emerging fast as a new era technology for transgenic development and precision breeding in cotton.

CRISPR/Cas 9 for cotton fibre development

Cotton fibre is the major economic product of cotton and the purest natural source of cellulose. In the past couple of decades, many scientists have been trying hard to understand the mechanisms with which cotton cells control fibre initiation during early developmental stages, so that the knowledge could be used to improve cotton fibre yield and quality. At present, many coding and noncoding genes, including several transcriptional factors (Mansoor and Paterson, 2012) and microRNAs (Wang and Zhang, 2015), have been identified that are associated with cotton fibre differentiation and development. However, the role and the extent to which each gene contributes to cotton fibre development is unclear and the precise molecular mechanism controlling this process is still unknown. Traditional transgenic technology can be used to over express an individual gene to study its function during cotton fibre development; based on which several studies showed that some genes play a role during cotton fibre initiation and early development (Lu *et al.*, 2018; Huang *et al.*, 2018; Hu *et al.*, 2016). However, overexpression of a gene or a few genes could result in non-specific effects and it is hard to inhibit single gene expression through traditional transgenic technology that makes it nearly impossible to study a gene whose expression is inhibited during cotton fibre development. CRISPR-based genome editing technology not only allows transgene insertion on a specific location of the chromosome, but also makes it possible to knockout an individual gene for any biological study, including fibre development. As we know that many transcription factors, including the myeloblastosis (MYB) and TEOSINTE-BRANCHED1/CYCLOIDEA/PCF (TCP), are associated with cotton fibre initiation and early development, CRISPR/Cas could provide opportunities for precision manipulation.

In our recent study, we have successfully identified more than 200 MYB transcription factors in cotton (He *et al.*, 2016). These MYB transcription factors are differentially expressed during cotton fibre development as well as in response to different environmental stresses (He *et al.*, 2016; Huang *et al.*, 2018). Amongst these, at least 36 were either preferentially or highly expressed in 20-day post anthesis (DPA) fibres and these MYBs were identified as putative secondary cell wall (SCW) regulators (Huang *et al.*, 2018). MYB212 is required for cotton fibre elongation by regulating sucrose transportation into expanding fibres (Sun *et al.*, 2019). MYB25-like is an R2R3 MYB transcription factor, which is expressed only during cotton fibre initiation, particularly at the age of -1 to 3 DPA; this stage is very important for epidermal cells differentiating into a long cotton fibre (Walford *et al.*, 2011).

In 2017, we employed the genome editing tool CRISPR/Cas 9 to knockout MYB25-like gene in cotton. This is the first report to use CRISPR/Cas 9 tool to successfully knockout out an endogenous gene in cotton. In this study, we designed a simple and quick genome editing protocol (Figure 1) to knockout an individual cotton gene (Li *et al.*, 2017). This strategy can be used to design one or more guide RNAs (gRNAs) that can be used to target an individual gene. The vector construction is pretty simple and can be done in about two days using the commonly used Golden Gate assembly tools. After the vector is mobilised into the Ti plasmid and transformed into *Agrobacterium*, the traditional gene transformation methods can be used to insert the gRNAs and Cas9 protein genes into the cotton cell. The gRNA and Cas9 protein function in the cotton cell to edit the individual gene. In most cases (Figure 1), it is possible to obtain genome editing mutants in about 6-9 months (Li *et al.*, 2017).

There are two copies of MYB-25 like genes: one from the A-subgenome and another from the D-subgenome. Using CRISPR/Cas 9, both the MYB25-like genes were targeted and knocked out (Li *et al.*, 2017). The genome editing efficiency is very high. There are various gene editing events, and both addition and deletion events existed in the genome editing process. The extent of nucleotide deletions depends on how many gRNAs work; a couple of nucleotides could be deleted when one gRNA worked or hundreds of nucleotides were deleted when two gRNAs worked (Li *et al.*, 2017). Our results show that knockout of MYB 25-like gene affects only cotton fibre initiation and no other cotton traits; compared with the wild type, MYB 25-like mutant did not show any difference except the fibre development (Figure 2). This suggests that CRISPR/Cas 9 is a powerful tool to study gene regulatory functions in cotton fibre development.

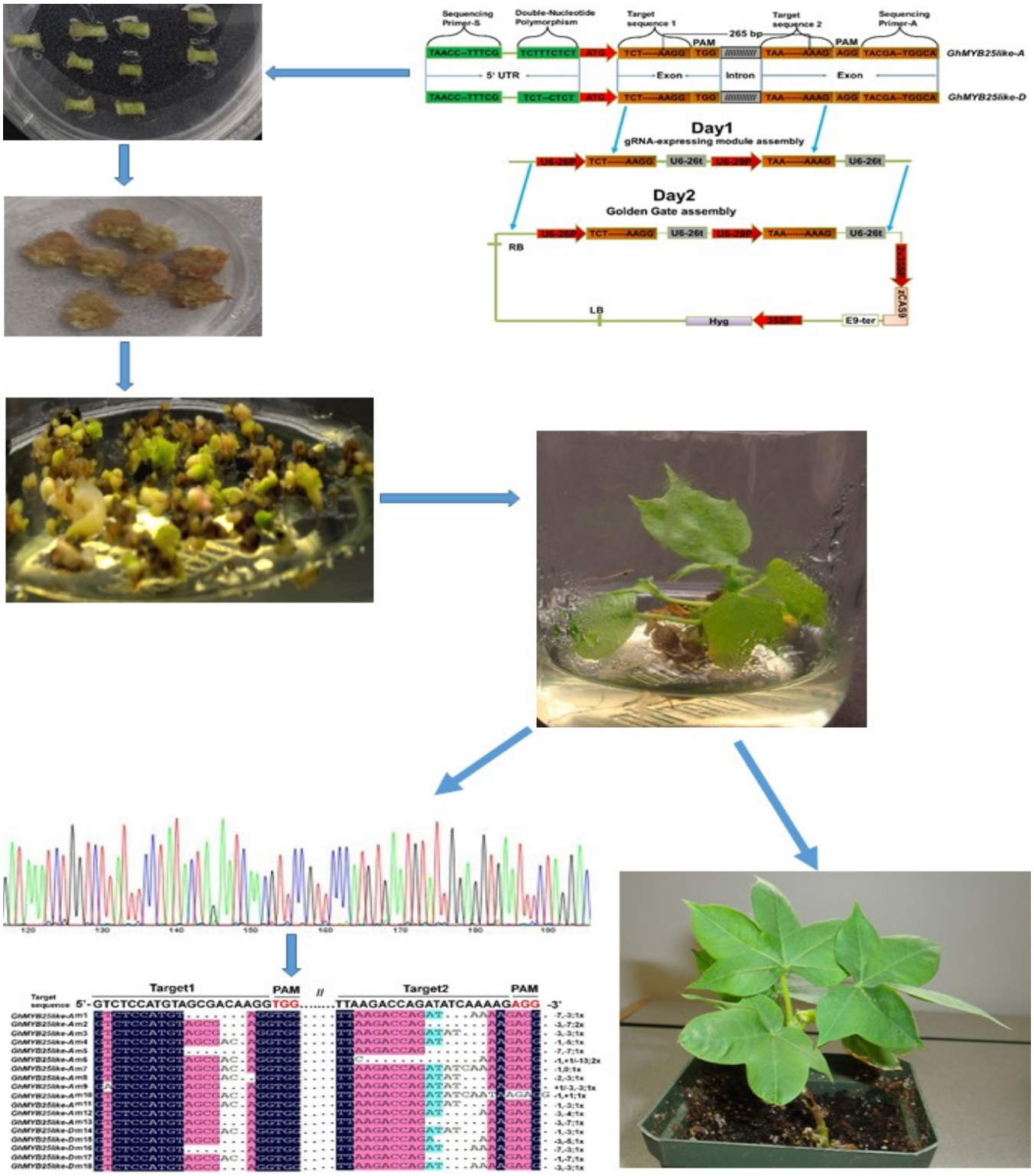

Figure 1. Diagram of CRISPR/Cas 9-mediated genome editing in cotton

Figure 2. Knockout of MYB 25-like transcription factor gene results in fibreless mutants in cotton.

During cotton fibre differentiation and development, certain 14-3-3 genes were found to be differentially expressed. The 14-3-3 proteins are a class of conserved proteins that widely exist in many organisms. Overexpression of 14-3-3L gene in transgenic cotton promoted cotton fibre elongation and further resulted in longer cotton fibre. In contrast, inhibiting the expression of three 14-3-3 genes by Ribo Nucleic acid interference (RNAi) inhibited fibre initiation and elongation in cotton (Zhou *et al.*, 2015). The *Alanine-Rich-Protein* (*alarp*) gene encodes a protein (ALARP) that is rich in alanine and it is preferentially expressed in cotton fibre. The CRISPR/Cas9 genome editing efficiency was 71.4-100% and 92.9-100% for ALARP-A and ALARP-D, respectively. The nucleotides changed from 55 bp deletions up to 99 insertions. Nucleotide deletions and insertions may occur simultaneously in many of the edited plants. The potential off-target effect of genome editing was first predicted and PCR was employed to amplify and sequence the off-target site to understand any potential off-target effects for the predicted off-target site (Zhu *et al.*, 2018).

CRISPR/Cas 9 for root development and response to environmental stress

Climate change and global warming are a big challenge for cotton and all crops. Climate change not only causes a harsh environment, including drought and salinity stresses, but also creates unsuitable temperatures for crop growth. The ill effects result in changes in the ecological biodiversity that affect beneficial organisms, insect pests and diseases, thereby impacting cotton growth and development. Proper root development is extremely important for crops to mitigate the effects of abiotic stress, particularly for soil-related stress factors, such as drought, salinity, strong winds and extreme temperatures.

Arginase is an important enzyme in plants, which competes with nitric oxide synthase (NOS) for arginine (ARG) substrate. The latter enzyme *NOS* catalyses the synthesis of nitric oxide (NO). It is well known that nitric oxide is an important regulator of root development in plants; increase of NO production results in more lateral and adventitious roots. Overexpression of ARG significantly inhibited NO accumulation in cotton root and thus decreased the formation of lateral roots in transgenic cotton (Meng *et al.*, 2015). It has been surmised that ARG plays an important role in root development and further impacts plant responses to different abiotic stresses. Recently, Wang and colleagues (2017) employed CRISPR/Cas 9 technology to knockout out the *ARG* genes using two individual single gRNAs. Their results showed that both gRNAs worked very well and highly efficient genome editing events were achieved. For T1 generation of genome knockout lines, irrespective of high or low nitrogen medium, the ARG knockout out lines showed significant increase in the number of lateral roots as well as the total root surface area (Wang *et al.*, 2017). Enzyme activity analysis showed that arginase activity was significantly decreased in the ARG knockout lines. Under high nitrogen conditions, the ARG knockout lines showed at least 25% and 52% increase in the lateral root number and the total areas of the lateral roots, respectively (Figure 3) (Wang *et al.*, 2017). A similar phenotype was also observed for the genome knockout lines under low nitrogen conditions. However, the NOS activity was significantly enhanced, accompanied with increased synthesis of nitric oxide (Wang *et al.*, 2017). This suggests that ARG knockout out lines have better root development that would enhance transgenic cotton to absorb more water and nutrients from the soil and enhance plant growth and development and respond better to various environmental stresses.

The 14-3-3 proteins not only play important roles in cotton fibre development but also in plant response to both abiotic and biotic stress, including drought and salinity stress and disease infection. CRISPR/Cas 9 genome knockout of two copies of the 14-3-3d genes showed higher resistance to *Verticillium dahliae* infestation compared to the wild-type plants (Zhang *et al.*, 2018). After 18 days of inoculation with 10^5 conidia/ml *V. dahliae*, the 14-3-3d knockout lines had significantly lower disease symptoms and lower disease index compared to the wild type CCR35 plants (Zhang *et al.*, 2018). The disease rate decreased from ~90% in wild type to ~30% in the genome edited lines; the disease index decreased from 50% to less than 20% and the total fungal biomass also decreased by 80% on the infected cotton leaves of the genome edited plants (Figure 4) (Zhang *et al.*, 2018).

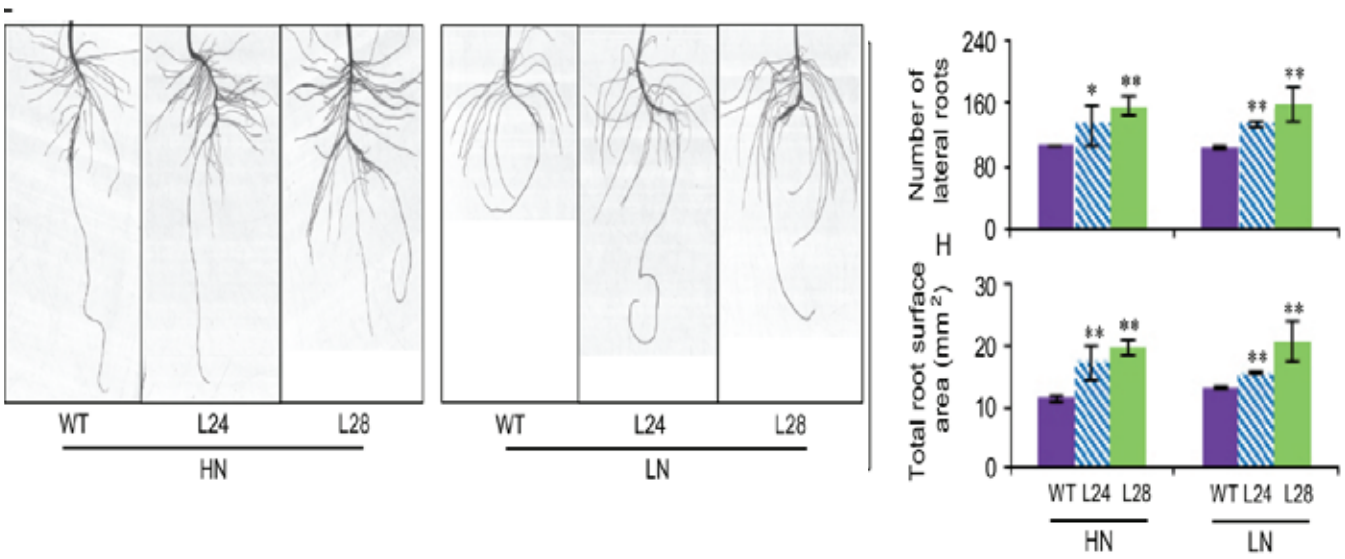

Figure 3. No matter under low (LN) or high (HN) nitrogen condition, ARG knockout lines (L24 and L28) were significantly increased the formation and total areas of lateral roots. Figures are adopted from a previous publication by Wang and colleagues (2017).

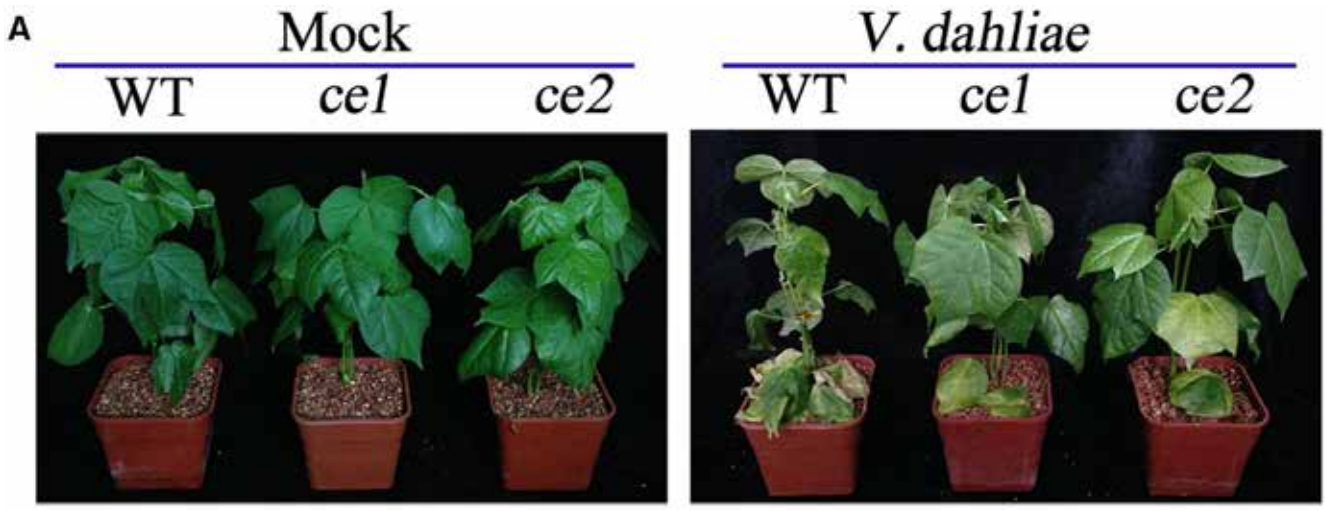

Figure 4. Genome knockout of 14-3-3d genes significantly enhanced cotton resistance to *Verticillium dahliae* infection. The photos were taken after 18 days of inoculation with 105 conidia/ml *Verticillium dahliae*. Ce1 and ce2 are two CRISPR/Cas9 genome editing lines. Figures are adopted from a previous publication by Zhang and colleagues (2018).

Future perspectives

CRISPR/Cas-based genome editing has a promising future for cotton breeding (Figure 5). This technology is simple with high desired target efficiency and least side-effects (Li *et al.*, 2017, 2018). In the short immediate future, this technology has the potential to quickly develop products that can move rapidly from the lab research to commercial cultivation. Research already provided a proof of concept that editing a single gene can significantly affect cotton fibre initiation and development. Without a doubt, as more genes associated with cotton fibre development are identified using multiple-omics methods, we can use the genome editing tools to modify these genes and allow the new varieties to produce better-quality fibre. At the same time, genome editing also has the potential to alter single genes associated with other agricultural traits, such as flower timing and development, root and leaf development to confer agronomic advantages to the plants.

With the burgeoning human population, the negative effects of climate change, global warming, and environmental stress factors are likely to get aggravated on cotton and agriculture (Piao *et al.*, 2019). Cotton crop is more sensi-

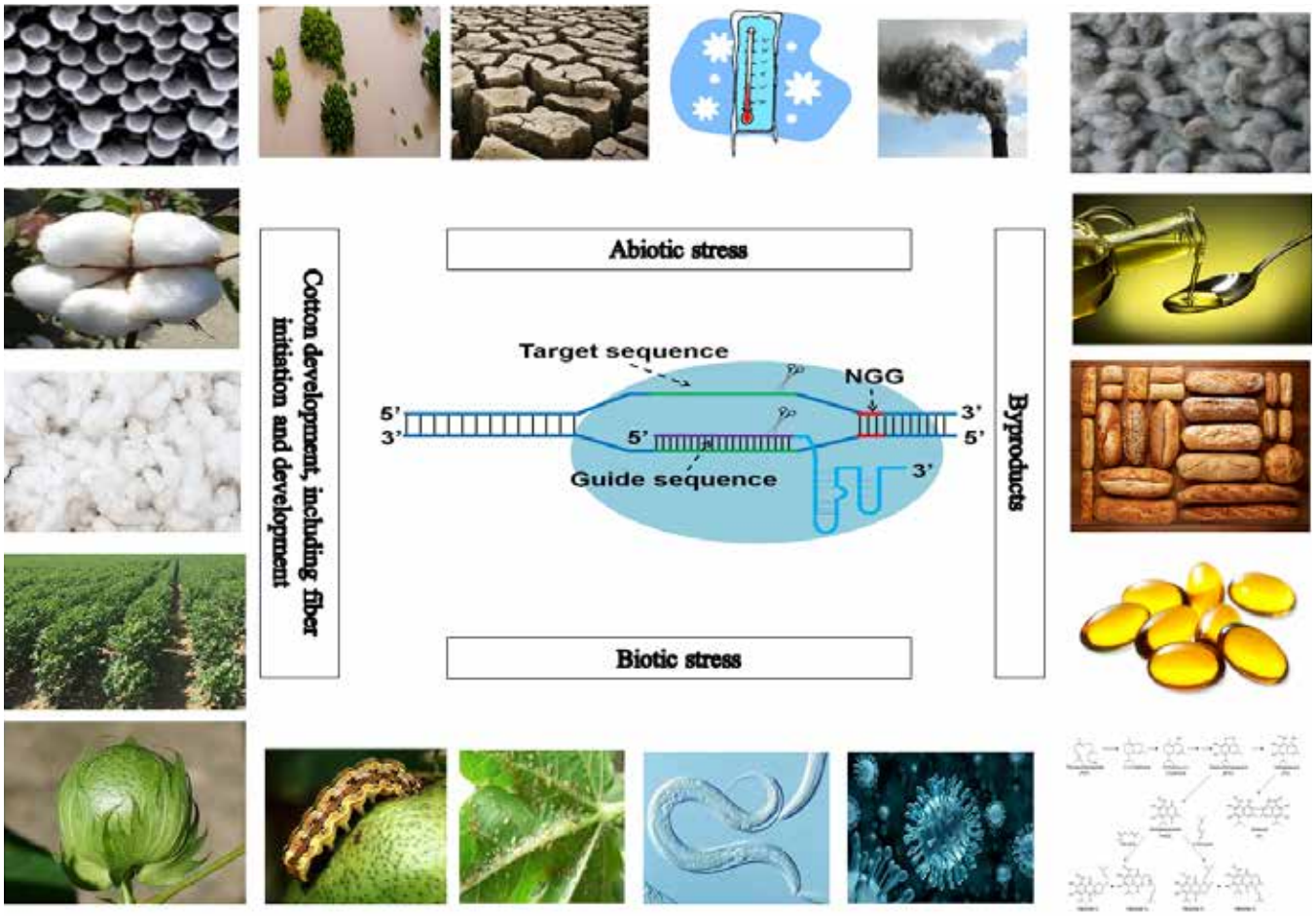

Figure 5. CRISPR/Cas-based genome editing opens a new era for cotton precise breeding

tive to severe environmental effects that include drought, disrupted monsoon patterns, altered biodiversity, salinity, flooding, hot and chilling temperatures, nutrient deficiencies, heavy metal pollution, and many other abiotic and biotic stress factors. Global warming also affects insect diversity and disease ecology that affects plant growth and development. All of these stress factors can significantly affect crop productivity and fibre quality. CRISPR/Cas-based genome breeding is likely to play an important role in creating new cotton cultivars for tolerance to these different stresses.

Cotton crop is most valued for its production of the most important natural cellulosic fibre. However, almost all parts of the cotton plant provide products that have economic value. In general, cotton seeds have 18%-26% oil, about 24% crude protein and are rich in Vitamin E. Regulating individual genes associated with the biosynthesis of oil, protein and vitamin E via genome editing, can allow the cotton cells to produce more of these products for human food and animal feed. The phenolic aldehyde pigment 'gossypol' which is unique to cotton is a toxic compound that is present in oil and seed-meal and presents a health hazard to monogastric animals. It is possible to develop varieties that produce gossypol-free seeds using CRISPR/Cas genome editing by knocking out an individual gene or a few genes that are associated with gossypol biosynthesis pathway. Thus, CRISPR/Cas genome editing technology opens a new era for precision cotton breeding for better cotton production..

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The ICAC Recorder

Mini-Review

CRISPR/CAS9, TALENS and Nucleases for Gene Editing in Cotton Improvement

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Introduction

During the course of civilisation, human beings gradually started learning and practicing agriculture to meet their daily dietary needs. Perhaps, since the advent of the prehistoric agriculture era, ancient farmers obliviously initiated crop improvement programmes by meticulously domesticating 'eye-catching' crop plants borrowed from the wild. With time, vintage sharp-minded agriculturists could unravel the science behind visible plant phenotypes and started applying their theories into farms and fields in the form of continual 'selection-breeding-selection' process. Gradually, plant breeders could comprehend the utmost importance and requirement of genetic variability as the fundamental prerequisite for selecting elite parents for a rewarding crop breeding programme. On the contrary, the long-term process of relentless human interventions toward large-scale domestication of crop plants has eventually narrowed down nature-borne genetic variability thus, creating a mounting crisis for desired breeding material in modern times. To mend this issue, plant scientists resorted to the use of man-made tools like induced mutation as one of the strategies to not only restore lost traits but also create such novel genetic variabilities that never existed in nature earlier (Sleper and Poehlman, 2006). Induced mutagenesis can be generated by both physical and chemical mutagens. Conventionally, radiation wave forms like X-rays, gamma rays, beta and ultraviolet irradiation, and neutrons etc. are used as physical mutagens whereas alkylating agents such as ethyl methanesulfonate (EMS) and methyl methane sulphonate (MMS) are the predominant sources of chemical mutagens (Scossiroli, 1970). However, mutations induced by those mutagens are utterly random with low frequencies which make the detection of desired mutants, rather cumbersome and time consuming (Micke *et al.*, 1990). Although mutagenesis could generate improved and/or novel monogenic traits, limited success has been achieved so far in case of complex polygenic traits like drought tolerance, yield, insect pest resistance, etc. (Maluszynski *et al.*, 2001). This is mostly due to the difficulty in screening quantitative traits, which at times undergo visually indistinguishable phenotypic changes and often escape the eyes of even expert plant breeders. Further, random mutagenesis may also be detrimental to non-target valuable traits resulting in marked reduction in their allele frequencies and even extinction from gene pool. For instance, during the course of domestication and breeding in maize, the gene *acyl-CoA: diacylglycerol acyltransferase* — responsible for producing oil rich in healthy monounsaturated fatty acid — mutated, resulting in a significant decline in oil yield (Zheng *et al.*, 2008). Moreover, there always remains a chance of severe health hazards even with minute breaches in safety protocols while handling mutagens. Meanwhile, unravelling of the plant's entire genome information led researchers to adopt a reverse genetics approach to develop novel DNA sequence based mutagens such as transposon, retrotransposon, T-DNA, TILLING (Targeting induced local lesions in genomes), etc. These tools could significantly enhance the precision level of mutagenesis including generation, detection and characterisation of plant mutants (Lucas *et al.*, 1995; McCallum *et al.*, 2000; Jeong *et al.*, 2002; An *et al.*, 2003; Mazier *et al.*, 2007; O'Malley and Ecker, 2010; Jiang *et al.*, 2015).

In contemporary times, the development of relatively inexpensive but high-throughput, whole-genome sequencing technologies and digitalised phonemics platforms have immensely empowered researchers to pinpoint trait associated genome loci and generate allelic arrays governing each target trait. Genotypic and phenotypic analyses of both wild-type and mutant populations have thus far generated huge volumes of information which unravelled innumerable novel genes and contrasting genome loci. However, the mere information of trait-specific putative genome targets is no good — unless there are tools designed exclusively to strike the bullseye with the lowest probability of off-target losses. Fortunately — with the advent of tools like meganucleases, Zinc Finger Nucleases (ZFNs), Transcription activator-like effector nuclease (TALENs) and most recently, clustered regularly interspaced short palindromic repeats associated system (CRISPR/Cas) — it is possible to virtually tinker with any desired gene or genomic loci, a technique popularly known as targeted 'genome editing' or 'genome editing with engineered nucleases' (GEN). These tools have demonstrated the ability to induce detectable targeted mutations in several plants and important crops as exemplified with *Arabidopsis*, to-

bacco, canola, potato, rice, maize, wheat, sorghum and cotton (reviewed by Weeks, Spalding and Yang, 2016; Li, Unver and Zhang, 2017). In addition to crop-improvement programs, GEN-mediated targeted mutations can be utilised for characterisation of novel genes to carry out basic studies in molecular biology. To carry out such studies, *Arabidopsis* and tobacco are preferably used as model plants due to their shorter life cycle and ease in handling large-scale progeny. On the other hand, cotton fibre — being a classical biological reference system for the study of single cell differentiation, development and cellulose biogenesis — undoubtedly qualifies cotton (*Gossypium spp.*) as a model plant in this context as well. Incidentally, cotton fibre represents the richest source of pure cellulose and fundamental raw material for the global textile industry.

Cotton belongs to the genus *Gossypium* consists of about 50 known species, of which four (*Gossypium hirsutum*, *G. arboreum*, *G. barbadense* and *G. herbaceum*) are cultivated globally (Campbell, Williams and Park, 2009). Primarily, cotton genetic improvement programs revolve around enhancement in fibre yield and quality together with resistance against insect-pests which are majorly regulated by complex multi-genes. During the last few decades, a significant decline in cotton genetic diversity has been observed (Boopathi and Hoffmann, 2016), which reflects the detrimental face of long-term domestication and recurrent breeding process. The narrow genetic base and continual genetic erosion of cotton are causing an alarming scarcity of improved alleles, which further restrict cotton breeders' ability to exploit the full potential of vital alleles governing economically important polygenic traits like fibre yield, abiotic and biotic stresses. Lack of suitable gene sources in the depleted cotton gene pool has compelled researchers to think beyond boundaries, which perhaps led to the application of cutting-edge transgenic technology in cotton. *Bt* cotton is a classic example where resistance source has been imported from a soil-borne bacterium to combat bollworms (James and Krattiger, 1996). Since its inception, transgenic technology has significantly improved cotton yield and farmer incomes. On the contrary, stable transgene integration and its optimum expression are the two obligatory pre-requisites for selecting a potential transgenic event, which demand cumbersome and time-consuming screening of thousands of putative events and further undergo strict scrutiny of environment biosafety regulatory bodies. However, recent reports on resistance development in bollworms against *Bt* cotton as recorded from several parts of India (Gujarat, Maharashtra and Telengana) (Kranthi, 2015), certainly indicate the diminishing performance of transgenic technology over time. Apparently, either transgenic technology needs an urgent upgrade or may be complemented by alternative innovative tool/s to justify its application in the near future. At this juncture, GEN-mediated targeted mutagenesis can be seen as a potent and sustainable option for cotton improvement, both quantitatively and qualitatively. These tools provide both transgenic and non-transgenic avenues for enhancing genetic variability and trait improvement in crops including cotton. In this mini-review, we will briefly discuss about various GEN tools, their applications and prospects in the area of cotton improvement.

Genome Editing with Engineered Nucleases

Over the past few decades, tremendous breakthroughs have been achieved in the area of developing innovative and practical genome editing tools. The potential applications of those tools are evidenced not only in animal systems but also in plants. The tools like Zinc Finger Nucleases (ZFNs), Transcription activator-like effector nucleases (TALENs) and Clustered regularly interspaced short palindromic repeats- CRISPR-associated system 9 (CRISPR/Cas9) are commonly used for targeted genome editing. In all these systems, an engineered nuclease is used in combination with a sequence specific DNA binding domain (ZFNs and TALENs) or a guide RNA (gRNA for CRISPR/Cas9) to induce double stranded break (DSB) at potentially any user defined target site/s. These DSBs then undergo a healing process orchestrated by the cell's inbuilt DNA repair mechanisms *viz.* error prone Non Homologous End Joining (NHEJ) and/or Homologous recombination (HR) mediated DNA repair pathways (Jackson, 2002). Both NHEJ and HR mediated pathways of DNA repair leads to mutagenesis which may result in loss or gain in function of target gene or even site-specific introduction of a novel gene sequence (Wright *et al.*, 2005).

First Generation Genome-Editing Tools

ZFNs and TALENs are among the first generation of genome-editing tools, accompanied by a customisable DNA binding domain fused with chimeric non-specific nuclease. A typical ZFN polypeptide comprises of two functional domains, a modular DNA-binding domain and a DNA-cleaving domain. The DNA-binding domain contains a characteristic alliance of 3-6 ZF ββα-folds that recognise 9–18 base pairs sequence of target DNA. Whereas, a DNA-cleaving domain contains nonspecific *Fok*I nuclease, a type IIS restriction enzyme (Kim, Cha and Chandrasegaran, 1996), fused with the C-terminal end of ZF DNA binding domain (Cathomen and Joung, 2008). As the enzymatic activity of *Fok*I nuclease is triggered only upon dimer formation (Smith *et al.*, 2000), a pair of ZFNs is designed in such a manner that each recognises and binds to two opposite non-palindromic strands of target DNA with a gap of 5 to 7 bp long spacer sequence (Bibikova *et al.*, 2001), thus facilitating an ambient interaction between two *Fok*I monomers forming an active dimer. Dimerised *Fok*I then introduces DSBs at user-defined target DNA sites which

subsequently lead to epNHEJ and HR mediated DNA repair, creating mutagenesis. ZFNs have been successfully used in the genome alteration of several plants *viz*. maize (Shukla *et al.*, 2009), Arabidopsis (Zhang *et al.*, 2010), tobacco (Schiermeyer *et al.*, 2019), soybean (Curtin *et al.*, 2011) etc. In addition, ZFN driven locus specific multiple transgene stacking was also established in wheat, thus proving its utility in trait stacking in crop plants in a fast yet simple way (Ainley *et al.*, 2013).

Quite akin to ZFNs, another genome-editing tool called transcription activator-like effector nucleases (TALENs), share almost an analogous working principle behind their individual modes of action. The DNA binding domain of TALENs is derived from a naturally occurring plant pathogenesis-related bacterial (*Xanthomanas*) protein called Transcription activator-like effector (TALE). Each TALE protein comprises of a highly conserved 33–35 amino acids repeat capable of recognizing a single base pair (bp) of DNA with the aid of two hyper-variable amino acid residues called repeat-variable di-residues (RVDs) (Gaj, Gersbach and Barbas, 2013). Converting this into an advantage, a complementary pair of TALENs is designed to harbour an array of nucleotide sequence specific RVDs to bind the opposite strands of target DNA separated by a spacer region of appropriate length (Christian *et al.*, 2010). Thus, like ZFNs, the architecture of TALENs typically contains a tailored DNA binding domain which guides the same *Fok*I endonuclease to target a predetermined genomic site. While, ZFNs require an array of linked 3-6 ZF repeat modules each recognizing a specific DNA triplet *via* ~30 amino acids, TALENs interact with single bp at the expense of merely two amino acids (RVDs). This enables the design and assembly of DNA binding domain of TALENs much simpler and less complex than the bulky ZFNs (Christian *et al.*, 2010; Li *et al.*, 2010).

Like ZFNs, TALENs have been successfully used to target several crop plants for inducing desired mutations as in *Arabidopsis* (Christian *et al.*, 2013), tomato (Lor *et al.*, 2014), rice (Ma *et al.*, 2015), barley (Budhagatapalli *et al.*, 2015), potato (Nicolia *et al.*, 2015) etc. TALENs mediated induction of heritable mutations was also demonstrated in two of the world's major world food crops; rice (Zhang *et al.*, 2016) and more recently in wheat (Luo *et al.*, 2019).

Next-Generation Genome Editor: CRISPR/Cas9

Among the various contemporary genome editing tools, "CRISPR/Cas9" (clustered regularly interspaced short palindromic repeats- CRISPR-associated protein-9) tool is gaining immense popularity primarily because of its low cost, ease of design and ability to target multiple genome sites without compromising target specificity. This tool is conceptualised from the bacterial adaptive immune system evolved to combat viral invasion (Barrangou *et al.*, 2007). Bacterial CRISPR locus is characterized by highly homologous short palindromic repetitive sequences (21-48 bp) interrupted by spacer regions (26-72 bp) containing oddments of previously invading viral genomes (Jansen *et al.*, 2002; Bolotin *et al.*, 2005; Grissa, Vergnaud and Pourcel, 2007; Rath *et al.*, 2015). When invaded by the same virus again, the CRISPR loci recognises the alien genome *via* acquired spacer sequence and cleaves the exogenous viral DNA aided by a non specific CRISPR-associated system (Cas) endonuclease. CRSPR-Cas9, a simpler modified version of this bacterial defence system adapted from *Streptococcus pyogenes* (Jinek *et al.*, 2012), is being preferably used by researchers for genome editing in several animals and plants which resulted in a considerable amount of success till date. The customized CRISPR/Cas9 system contains CRISPR sequence harbouring a user defined spacer sequence (~20 nucleotides), a region homologous to the genomic site to be modified. The transcription of CRISPR sequence yields an adapter RNA, called small guide-RNA (sg-RNA) which contains a hairpin loop at its 3' end and an unzipped 5' end harbouring spacer sequence. While the hairpin loop carries the Cas9 endonuclease, the spacer sequence of sgRNA binds the complementary sequence of target genome site, protospacer by Watson-Crick base pairing. The protospacer region is then recognized by Cas9 with the assistance of a specific downstream localised 3 nucleotide signature motif (5'-NGG-3') known as Protospacer Adjacent Motif (PAM). Cas9 components RuvC and HNH then precisely cleave each of the two strands of the target DNA (Jinek *et al.*, 2012).

The key advantages that CRISPR/Cas offers compared with the other contemporary genome editing tools like ZFNs and TALENs are:

- S**impler design:** Unlike ZFNs or TALENs which count upon the complicated interaction between engineered protein dimer and DNA, CRISPR/Cas requires only a single monomeric nuclease and a customized guide RNA for genome editing. This makes the design of the tool easier, more affordable and more flexible.
- **Specificity:** The specificities of TALENs and ZFNs in particular, are contextual as there are equal chances that they may cleave off-target sites which are partially identical to the desired target site (Cheng *et al.*, 2011; Mussolino *et al.*, 2011). In contrast, the target specificity of CRISPR/Cas9 system is governed by a simple yet robust Watson-Crick RNA-DNA complementary base pairing which is more specific than protein-DNA interaction.
- **Ease of delivery:** CRISPR/Cas9 assembly can be delivered to the target cell *via* T-DNA or particle bombardment mediated customized plasmid construct or in the form of pre-assembled vector-free protein-ribonucleotide (Cas9:sgRNA) complex (Kanchiswamy, 2016), as per demand of the relevant experiment.

- **Multiplexed genome editing:** Using custom-designed Cas9:sgRNA complex, virtually any genomic site can be targeted with high specificity, which makes the tool more powerful than other parallel genome editors. Moreover, multiple pre-determined genome sites can easily be targeted at once, just by the co-delivery of customized multiple sgRNAs and a single Cas9 without compromising target specificity (Lopez-Obando *et al.*, 2016). This feature provides an excellent opportunity for the improvement of complex qualitative traits like insect-pest resistance, drought and yield in crop plants. Remarkably, a study in *Arabidopsis* shows simultaneous targeting in as many as fourteen distinct genome sites using CRISPR/Cas9 with no detectable off-targets (Peterson *et al.*, 2016). Similarly, multiple genomic sites have been targeted using CRISPR/Cas system in other crop plants such as tomato, *Brassica* and cotton. (Li *et al.*, 2018; Ma *et al.*, 2019; Wang *et al.*, 2018)

Genome Editing in Cotton, Current Status and Future Prospects

Since their inception, use of the modern state-of-the-art GEN tools have been primarily restricted to those group of plants that possess less complex diploid genomes and readily respond towards transformation and regeneration. Poor regeneration potential via somatic embryogenesis in cotton creates a major bottleneck for the practical application of GEN tools. Moreover, two of the ruling commercially cultivated cotton species — *Gossypium hirsutum* and *G. barbadense* — are allotetraploid in nature which further hinders the targeted modifications in all the four redundant alleles of a gene at once. Considering all these limitations, contemporary cotton researchers have been focusing primarily on the assessment and validation of the efficiency and reliability of GEN tools prior to large-scale application in cotton improvement. In this context, D'Halluin and co-workers were the first who demonstrated the potential of an engineered meganuclease for targeted genome editing in cotton, which resulted in introgression of heritable multiple traits (Herbicide resistance and insect resstance) (D'Halluin *et al.*, 2013). Based on the results, the authors concluded that GEN tools may be potentially used for a non-transgenic mode of targeted gene pyramiding in plants. Due to the ease of design and target specificity, the efficacy of CRISPR/Cas system has been tested in some recent studies pertaining to cotton. In one such study, CRISPR/Cas9 mediated gene silencing of green florescent protein transgene was observed due to targeted mutagenesis mediated indels.

However, the study also emphasised the importance of prior examination of sgRNAs for identifying a suitable protospacer region before targeting the gene of interest to avoid any off-target DSBs (Janga, Campbell and Rathore, 2017). Despite the genome complexity of allotetraploid cotton (*Gossypium hirsutum* L.), it was possible to achieve targeted mutagenesis in two pre-intended sites representing both A and D sub-genomes simultaneously, which resulted in the manipulation of *GhMYB25-like A* and *GhMYB25-like D* genes with whooping mutation frequencies of 100% and 98.8% respectively, without any traceable off-target effects (Li, Unver and Zhang, 2017). This certifies the potential of CRISPR/Cas9 tool for its robust application even in crops with complex genomes as exemplified in hexaploid wheat as well (Wang *et al.*. 2014). In a similar way, multiple gene (one endogenous gene *Cloroplastos alterados 1* and a transgene *Discosoma red fluorescent protein2*) were edited with an efficiency of 66.7%–100%. The altered phenotypic variation was inherited in the subsequent generation and carried no detectable off-target effects (Wang *et al.*, 2018). In another study, it was evidenced that CRISPR/Cas9 tool could efficiently alter cotton *Cloroplastos alterados 1* and *vacuolar H+-pyrophosphatase* genes at user defined sites with mutation frequencies ranging from 47.6–81.8% (Chen *et al.*, 2017). Although no unintended mutations were traced from the 30 out of 64 potential off-target loci examined in the study, the authors could not rule out the possibility in the remaining 34 loci. This apprehension of CRISPR/Cas9 associated off-target mutagenesis may apparently arise due to some earlier reports which recorded side effects with this tool (Xie and Yang, 2013; Bortesi and Fischer, 2015).

However, a study conducted last year (Zhu *et al.*, 2018) had demonstrated editing of the cotton fibre gene *ALARP* using CRISPR/Cas system inducing targeted mutation frequencies ranging from 71.4-100% with no traceable off target mutations. Thus, it is very much apparent from above mentioned gene editing reports in cotton that CRISPR/Cas9 system has enormous potential for future cotton improvement programs. The recently sequenced genomes of cotton species have predicted a heap of coding sequences with unknown function. Therefore, a highly competent and target specific tool like CRISPR/Cas9 provides solid opportunities for its deployment in accelerated unravelling of functional genomics which would form the practical basis for gene editing for cotton improvement.

The primary goal of cotton improvement programs revolves around its fibre quality and yield. Literally speaking, fibre is an exclusive signature product of a cotton plant and has its own unique developmental pathway. Insights for developmental clues other than fibre development in cotton can be understood by studying the parallel processes in model plants like *Arabidopsis*, tobacco etc. On the other hand, it is extremely crucial to have deeper insights into fibre development for improving cotton fibre yield and quality traits. Unfortunately, only limited progress has been made to decipher the genetic regulatory mechanism underlying fibre cell patterning, differentiation and devel-

opment. However, besides genetic factors, high throughput genomics and transcriptomics could unravel several epigenetic factors governing cotton fibre development (Song *et al.*, 2015; Wang *et al.*, 2016). Although the first generation GEN tools like ZFNs and TALENs can also target virtually any given gene/s, but inducing targeted modifications in the epigenetic loci is beyond their limits. On the other hand, CRISPR/Cas9 is reported to be equally efficient in targeting and cleaving methylated DNA sequences too (Ding *et al.*, 2013; Hsu *et al.*, 2013; Bortesi and Fischer, 2015). Clearly, CRISPR/Cas9 technology provides a unique opportunity to explore the functions of multiple genes at one go, whereby cotton biologists can rapidly unearth the whole biological network specifying a developmental process, including fibre development in cotton. Therefore, CRISPR/Cas9 shows great promise for cotton improvement, not essentially limited to functional genomics but for characterisation of any desired plant developmental pathways.

CRISPR System Based Base Editing Technology ('Base Editor')

Base editing technology is an emerging, updated version of CRISPR based genome editing approach, which amalgamates CRISPR components with other enzymes to precisely induce point mutations into cellular nucleic acid (DNA/RNA) without causing any double-stranded breaks (DSBs) (Komor *et al.*, 2016, Raees and Liu, 2018). DNA base editors are basically engineered with the fusion of CRISPR with a catalytically inactive nuclease and a cytidine deaminase enzyme which enables direct conversion of C to T (or G to A) in the target genomic site. This tool has been reported to be highly precise with its significant ability to rectify specific point mutations that are detrimental to human health (Komor *et al.*, 2016). Of late, this base-editing technology has been applied in cotton resulting in the successful introduction of single-base mutations with at least one C→T substitution at three target sites from two target genes, *GhCLA* and *GhPEBP*. Moreover, there were no off-target effects noticed while subsequent inheritance of edited bases were noticed in the T1 progeny as well (Qin *et al.*, 2019). This undoubtedly paves the way forward towards a better, efficient and more precise way of genome editing options in the near immediate future with least off-target side effects.

Conclusion

Precise, targeted mutagenesis was just a dream for conventional cotton breeders until the inception of genome editing tools, particularly CRISPR/Cas9 system. Of course, like any other impressive technology, CRISPR/Cas9 tool also offers other advantages, as well as some limitations. The major concern expressed by regulatory authorities is the possibility of CRISPR/Cas9 associated low-frequency, off-target mutagenesis. However, the benefits of this robust, easy to deliver, relatively cheap and highly precise genome editing tool more than overcomes the associated occasional minor pitfalls. Moreover, the CRISPR/Cas9 tool also offers the flexibility of opting for a non-transgenic method of genome modification (Kanchiswamy *et al.*, 2015; Zhang *et al.*, 2016 Rimsha Farooq, *et al.*, 2018; Shew *et al.*, 2018), which bypasses the hurdles of strict bio-safety scrutiny. Perhaps the imperfections in genome editing tools like ZFNs, TALENs and CRISPR/Cas9 provide wider scope for researchers to not only improve existing tools, but also to innovate better tools which will ultimately nudge crop improvement programs to the next level. As far as the next generation cotton improvement is concerned, as the existing transgenic era seems saturated and fatigued, it is now the perfect time to introduce 'next-gen genome editing tools', not to replace but to complement the existing transgenic technology and conventional breeding tools for sustainable cotton improvement.

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The ICAC Recorder

Mini-Review

Biosafety Regulation of CRISPR/Cas9 and Genome-edited Crops

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Gene editing technologies such as site-directed-nucleases (SDNs) and oligonucleotide-directed mutagenesis (ODM) are recent additions to the wide array of genetic modification tools. Amongst these, the clustered regularly interspaced short palindromic repeats (CRISPR) and *CRISPR*-associated protein-9 nuclease (*Cas9*), together referred to as CRISPR/Cas9, have emerged as the most popular tools for gene-editing.

In the process of domestication, crop plants have been continuously subjected to genetic changes to suit human dietary preferences and animal feed requirements. Several of the following methods cause genetic changes in plants:

- Conventional plant breeding techniques involve the exchange of large segments of chromosomes between plants.
- Mutation breeding results in random genetic mutations and highly unpredictable changes.
- Transgenic transformation results in trans-gene insertions at random locations of plant's genome.
- Ribonucleic acid interference (RNAi) silences target genes and homologous genes with similar sequences, however with a fair probability of causing off-target gene silencing as well
- Site-directed nucleases have the potential to edit precise targeted locations on the gene but have also been reported to cause unexpected off-target effects.

The above technologies cause heritable changes which are irreversible, which is why regulating the processes of genetic engineering (GE) and biosafety assessment of the GE products assume paramount importance to ensure that the changes do not have irreversible negative consequences to the environment and human welfare. In general, biosafety regulations for gene-edited products aim to critically examine unintended genetic and epigenetic changes for any potential risks to human health and environment. Gene-edited plants are likely to be very different from transgenic plants with respect to the traits and unintended effects. Therefore, the regulatory guidelines must be differently formulated. However, regulatory guidelines to assess the biosafety of gene-edited plants have not as yet been properly developed. In general, many countries have been evaluating potential biosafety risks of gene-edited crops using the same parameters that are used to assess the biosafety of transgenic crops (Wolt, 2017; Eckerstorfer *et al.*, 2019).

The following site-directed-nuclease based technologies are most commonly used for genome editing in crops and animals.

- Clustered regularly interspaced short palindromic repeats/CRISPR associated nuclease 9 (CRISPR/Cas9)
- Oligonucleotide directed mutagenesis (ODMs),
- Engineered Meganucleases (EMNs),
- Zinc Finger Nucleases (ZFNs), and
- Transcription Activator-like Effector Nucleases (TALENs).

These techniques open the possibility for targeted gene modification or alteration of nucleotides in an existing molecule of DNA or RNA, as well as insertions or deletions of large sequences in specific target locations (Agapito-Tenfen*et al* 2018). Among the new gene-editing systems, CRISPR/Cas9 tool has emerged as the most powerful of all genome editing technologies available currently and has sparked a new revolution in biological and agricultural research. CRISPR/Cas9 technology is widely acknowledged as a powerful tool that has the potential to create genetic variability in a precise and targeted way, representing a new era in crop breeding. The system is versatile, fast and inexpensive, allowing genome editing strategies to be more accessible and efficient compared to other technologies such as the Zinc Finger nucleases, TALENs and other first-generation genome editors. However, though the CRISPR system edits genes to create a single point mutation or even repair a mutation in the gene thereby enhancing or repressing gene expression, undesirable off target effects can also be expected to a certain extent. With the development of the high fidelity mutant Cas9 nucleases, the editing specificity has been reported to increase with negligible or no off-target effects. Undoubtedly the precision and convenience of CRISPR/Cas9 system will make it one of the most desired techniques of gene-editing in both mammalian and plant species for next few decades.

The potential of gene-editing in plant improvement extends to a wide range of plant species such as wheat and cotton which have a complex genome that makes plant

breeding very complicated. CRISPR based technologies have been found to be significantly efficient in editing genes in a precise manner in several organisms including many agriculturally important crop species to enhance trait values in crop protection and crop production. Gene-editing has been used to develop herbicide tolerant plants in cotton (D'Halluin *et al.*, 2013), rapeseed, potato, rice, flax, maize, cassava, tobacco and soybean. It is unlikely that compared to transgenic HT crops, the gene-edited herbicide resistant crops could pose any different concerns on weed resistance to herbicides. CRISPR/cas9 tool have been used to induce targeted mutagenesis in cotton to fine-tune several traits including fibre quality improvement, disease control and insect pest management. For instance, CRISPR/Cas9 based genome editing could successfully control the dreaded cotton leaf curl disease (Iqbal *et al.,* 2016). Likewise, the pheromone odorant receptor OR16 gene was knocked out using CRISPR/Cas9 to disrupt mating in the bollworm *Helicoverpa armigera* (Chang *et al.,* 2017).

The possibility of improving economically important traits in agriculture through CRISPR based genome-editing presents an opportunity for rapid and inexpensive creation of genetically improved gene-edited crop varieties and enhancement of genetic diversity in plants. Amusingly, unlike transgenic technology, tailored *CRISPR/Cas9* cassette is capable of creating heritable alteration at any target sites of hosts' genomes even upon their mere transient expressions. Therefore, this means that such genome edited crops may be exempted from being tagged as "transgenic or GMOs" as *CRISPR/Cas9* genes eventually gets eliminated in the subsequent generation. The CRISPR complex constitutes a genome-editing machinery comprising of a guide-RNA (gRNA) and Cas9-nuclease. The CRISPR genes accompanied with expression promoters can be introduced as a cassette into the cell to edit the target gene sequences in the genome either after getting integrated into the host genome or by transient expression of the components without getting integrated into the genome. Integration of the CRISPR genes into the host genome will however, turn the product into a GMO. Thus, the target genes can be edited either by stable introduction of recombinant constructs or transgenes into the plant genome or as transient introduction of either transient DNA constructs or synthetic-oligonucleotides or functional ribonucleoproteins into the target plant cell (Kanchiswamy, 2016). In the transient introduction of the editing cassettes, the 'CRISPR complex' gets eliminated from the progeny plants of the subsequent generation, while edited traits continue to be inherited in a stable manner.

Proper deployment of the gene-edited crops will largely depend on biosafety regulation. Biosafety guidelines will have to focus on the possible risks that the genome edited products may pose to the environment and human safety while encompassing regulations of all aspects of the processes involved in the product development. While regulation of developmental processes in containment mainly relates to 'good laboratory practices', the gene-edited crops need careful assessment for potential off-target effects in the target organism and any possible risks of unintended genetic changes in non-target organisms and the risk potential for adverse consequences to the environment and human welfare. There is evidence that the CRISPR technology can also introduce off-target effects. Thus, there is a need to assess and regulate unintended consequences and off-target effects through biosafety regulatory mechanisms.

Some of the basic aspects of regulation at the developmental stages must consider the following processes and protocols that are used in gene-editing.

- The type of the organism (humans, animals, plants, microbes etc.,) and the kind of target tissues (embryos, germ-line cells, cell cultures, cells or whole organisms) used
- The target genes, their primary role and functional significance
- Target gene sequence and bioinformatic information of its homologous genes in other related organisms
- Mechanisms with which the CRISPR genes (gRNA and Cas9) are delivered into cells or tissues (viral vector, liposome, plasmids, nanoparticles, injections, syringes etc.,) and justification of the deployment
- Purpose and objectives of the gene-editing and predicted effects
- Observed off-target site modifications and the resultant traits
- Gene drive experiments, editing strategies and engineering containment protocols, if applied and precautions taken to prevent environmental escapes and
- Envisioned bioethical, socio-economic and societal consequences

One of the major issues with gene-editing lies with the biosafety regulations, which are yet to be developed in many countries. The question remains whether gene-edited crops will also be governed under the same regulations as those of conventional GM crops or would need a different set of biosafety regulations. The major concerns with transgenic plants were that "*insertion of foreign DNA (DNA from other organisms) to plants may have deleterious effect to human health and environment in the long run*" and "*insertion of T-DNA along with antibiotic resistance genes could have negative effects*". However, with a proper selection process, and with the recently developed high fidelity Cas9, the CRISPR edited plants can produce transgene free offsprings, with the least possibilities of resultant off-tar-

get effects, which should have no issues for health safety, should satisfy anti-GMO activists and bypass the current biosafety regulation (Lee *et al.*, 2018).

The current risk assessment framework was developed for products of classical GM techniques. European Food Safety Authority holds that products developed using Site-Directed-Nuclease-3 techniques (insertion by homologous recombination) would be categorized as GMOs and regulated under EU Directive (Sprink *et al.*, 2016). The European Court of Justice points out that the new and emerging gene-editing techniques lack a long-history of safe-use in any organism. Indeed, scientific literature reveals that the biotechnology community is still focused, at a fundamental level, on improving the efficiency and the applied uses of such techniques. The key question is how to regulate the safe use of new gene-edited products. Several aspects of the current framework and its implementation stand to benefit from reconsideration in light of progress in the broader field. Examples of these aspects include: choice of test organisms for identification of on-target and off-target effects; use of the whole edited plant/derived product as stress or in effect-testing; and expansion of the repertoire of molecular techniques to include omics in molecular characterization of hazards (Casacuberta *et al.*, 2015). In particular, the risk assessment guidance may need to be revised to enhance suitability for evaluating impacts of products by new and emerging gene-editing techniques on environmental, human, and animal health (Agapito-Tenfen*et al.,* 2018).

For transgenic crops, the Cartagena Protocol (CPB) held on January 29, 2000 under the aegis of 'Convention of Biological Diversity (CBD)' was instrumental in producing a globally harmonized regime for biosafety. The protocol mainly sought to protect biological diversity from the potential risks posed by Living Modified Organisms (LMOs). The CBP resolution was ratified by 170 parties including European Union (EU) and UN countries and is now adopted by more than 135 countries.

However, for gene-edited crops there is no internationally accepted harmonized regulatory framework thus far. Different countries have evolved different guidelines for research on gene-editing and assessment of gene-edited products. There has been an intense debate on whether gene-editing can be classified under genetic engineering or could be viewed differently from a regulatory perspective for research and biosafety evaluation. While transgenic crops contain foreign genes that are inserted into the host chromosomes, gene edited crops contain genes that are edited by insertions or deletions. CRISPR/Cas9 and other target-genome editing methods also require the introduction of genes into the target cells and therefore the process qualifies to be called as genetic engineering. But the key difference would be whether the introduced genes are integrated into the host genome or whether they only perform gene-editing by maintaining their status as extra chromosomal elements and are not inherited. Clearly, if the CRISPR/Cas9 genes are not integrated and inherited, the resultant gene-edited crop could be seen as modified or improved but identical to the natural crop. In such cases where the CRISPR/Cas9 genes are integrated and inherited, the genetic engineering process would be under intense scrutiny of laboratory safety procedures and the product would be spared from the rigorous environmental biosafety evaluation and assessment.

In the United States, genome-edited plants are exempted from regulations covering GMOs if they are not produced by transferring DNA from other species and are indistinguishable from the plants developed through conventional breeding. However, in 2018, the Court of Justice of the European Union (ECJ) ruled that CRISPR edited plants should be subject to the same stringent regulations as conventional genetically modified (GM) organisms. Different countries have been reacting differently to gene-edited crops. The food and drug administration of Iran decided to label genetically edited foods, similar to genetically modified foods to address their consumer concerns. In 2019, the Australian Government declared that it will not regulate the use of gene-editing techniques in plants, animals and human cell lines that do not introduce new genetic material. Previously, the CRISPR/Cas9 was governed by the same rules as GMOs and required the approvals by the Office of the Gene Technology Regulator (OGTR). Like many countries in Asia, China and India are yet to declare their policies of regulating CRISPR generated crops. It remains to be seen how different countries view the technology.

Crop improvement has entered a very interesting stage in the history of agriculture. Scientific advancement facilitates precise identification of specific gene sequences and genetic elements that regulate economically important traits in crops and also provides the tools which can be used to edit and modify specific target gene sequences with high precision, so as to direct the organism in an ordained manner as desired by the scientist. These changes are genetic and are inheritable. The changes are permanent and irreversible. There can be several ways of looking at the gene-editing phenomenon. The cell is a marvelous creation of nature that functions as well-oiled machinery guided by genes whose functions are inter-related. No gene functions as an island. All genes work in synchrony and harmony much like orchestrated musical notes in a symphony. From a short-term perspective, scientists focus on the necessity to alter gene sequences and modify organisms to suit requirements of the growing population. From a macro-perspective, fundamental questions must be asked as to why had a gene sequence evolved the way it did in the first place, and would the organism recognize the edited genetic change to repair it back to its original form, because probably that was the way that nature had

designed the gene for its well-being. It would be interesting to see how nations evolve regulatory guidelines in the future to address these issues from short-term and macro perspectives to ensure a better and sustainable future for the environment and mankind.

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The ICAC Recorder

Mini-Review

Cotton Fibre Quality Management for Sustainable Textile Industry

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Abstract

Cotton fibre is a subject of great interest for scientists and industrialists across the world due to its importance to the textile industry. Fibre quality is attributed to its various characteristics including fibre length, strength, micronaire value, colour, etc. Alteration of one or two of these traits can be interesting to evaluate their impact on quality but, being a multifactorial trait, it is necessary to use a combinational approach of genetic modification so as to breed all the desired alleles together to gather as many traits as possible for high-quality cotton fibre. Moreover, the use of genome editing technologies can also be very useful to knockout the traits responsible for inhibiting the expression of fibre related genes. The current study highlights important aspects of genome-based strategies for cotton fibre improvement and their application in detail.

Cotton from Plant to Fabrics

Cotton is one of the most significant crops worldwide. It is a major source of raw material for the production of textile products, cotton seeds (which are used as highly nutritional feed for livestock) and cotton seed oil (which is used in soap making and cooking). Cotton is a member of the family Malvaceae, which contains at least 50 species under the genus Gossypium. Amongst these, only four species are commercially used — *Gossypium arboreum, Gossypium herbaceum, Gossypium hirsutum* and *Gossypium barbadense* (Khan *et al.*, 2016; Iqbal et. al., 2001).

Cotton Fibre Development

Development of cotton fibre starts from single cell ovule in four complex overlapping stages:

- Initiation,
- Elongation,
- Primary cell wall synthesis, and
- Secondary cell wall synthesis (Basra & Malik, 1984).

The staple length and fineness of cotton fibre play a very important role for its utilisation in the textile industry (Bajwa *et al.*, 2013). Cotton yield is very important for the cotton growers and this can be achieved by increasing the number of fibres produced on the developing ovules (Xiao *et al.* 2016).

Various factors affect cotton fibre development. Some of these are summarised in this article.

Effects of Abiotic Stresses on Fibre Development

Abiotic stresses affect the overall quality and yield of fibre. The optimum temperature for cotton leaf, stem growth, fruit and seed development is 23.5-32°C. Cellulose formation during fibre cell wall synthesis is slowed down if temperature is decreased (Zheng *et al.*, 2012). During early stages of fibre development, it also inhibits the axial growth (Qiu *et al.*, 2007), whereas Schrader *et al.* (2004) reported that increase in temperature above 38°C will inhibit photosynthesis.

The regular functions of a cotton plant such as metabolism and turgor potential decreases when soil water contents are limited (Wang *et al.*, 2016). Drought, therefore, imparts negative effects on both quality and yield of cotton fibres. There are several studies which showed that water stress not only alters the fibre quality and cotton yield, but also reduces weight and number of cotton bolls (Lokhande and Reddy; 2014). Increasing water supply during square and boll formation stages is reported to enhance boll numbers, boll size and fruit-bearing branches. It has been reported that extreme conditions such as low rains reduce cellulose accumulation and impair photosynthesis while excess rains with cloudy weather decrease photosynthesis and thus fibre quality (Sawan, 2017). Fibre length is also reduced due to nutrient deficiencies (Lokhande and Reddy; 2014).

Genes and Transcription Factors Responsible for Fibre Development

Over 90% of lint is obtained from upland cotton (*G. hirsutum*) but its major disadvantage has been the relatively lower fibre quality in comparison to the superior extra-long-staple (ELS) cotton (*G. barbadense*). Genetic improvement plays a key role in meeting this agricultural challenge. Studies reveal that thousands of genes are responsible for fibre development (Fig. 1). Modern biotechnology focuses on introduction of more than one gene from different sources to one target (Khan *et al.*, 2016; John & Keller, 1996; Iqbal *et al.*, 2016; Ahmed *et al.*, 2018).

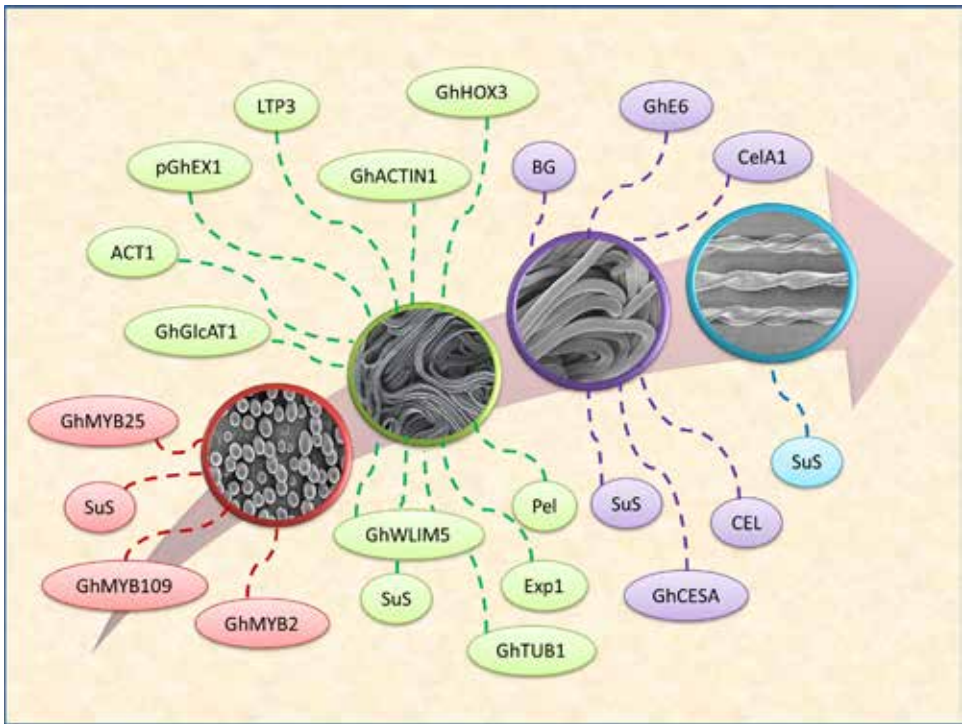

Figure1: Genes involved in development of cotton fibre

Epidermal cells of ovules are the source of single cellular cotton fibre origination; as a result, alterations in the genetic makeup of these epidermal cells can improve the quality of the originating fibres (Shi *et al.*, 2001). Genes are controlled by master regulators known as transcription factors (TFs). (Pati *et al.*, 2006). GhMYB25-like transcription factors are very important in fibre cell initiation and differentiation whereas GhMYB25, GhMYB109 and GhMYB2 transcription factors play significant role in fibre development (Huang et al., 2013).

GhHox3 genes play a vital role in production of fine-quality elongated fibres (Shan *et al.*, 2014). GhWLIM5 gene belongs to cotton LIM-domain proteins which have the ability to bind with actin cytoskeleton and bundling of F-actin filaments, therefore these are reported to be widely expressed in fibre elongation (Li *et al.*,2013). GhHOX3 is involved in the cotton fibre elongation and its silencing results in 80% reduction in fibre length (Shan et al., 2014). Pectin methyl esterase enzyme (PME) plays an important role in different developmental stages of fibre (Li *et al.* 2016). Plant LIM proteins have been reported as one of the key actin-binding proteins (ABP's) (Thomas *et al.*, 2007). The nuclear LIM-domain proteins act primarily in tissue-specific gene regulation and cell fate determination, whereas the cytoplasmic LIM-domain proteins function mainly in cytoskeletal organisation (Han *et al.*, 2013). It has been reported that GhLIM5 protein has a role in actin filament bundling (Li *et al.*, 2013) and down-regulation of the *GhACTIN1* gene resulted in shortening the length and weakening the strength of cotton fibres (Li *et al.*, 2005). Therefore, it is surmised that over-expression of *GhACTIN1* and *GhWLIM5* genes would result in increased fibre length and strength respectively.

In cotton, the developing cotton boll acts as a sink and breakdown of sucrose into its component hexoses and is the first step for utilisation of sucrose for fibre development. Sucrose cleavage is essential as it results in production of UDP-glucose (Li *et al.*, 2019). Sucrose synthases (SuS) and invertases control the degradation of sucrose in most plants (Shua *et al.*, 2009). Invertases hydrolyse sucrose into fructose and glucose (Gou *et al.*, 2007; Kleczkowski, 2010; Brill *et al.*, 2011). UDPG plays a vital role in cytosolic formation of sucrose and synthesis of polysaccharides (e.g. hemicelluloses) and pectin, and components of cell wall (Amor *et al.*, 1995; Gibeaut, 2000; Johansson *et al.*, 2002). Studies have revealed that decreased SuS activity at fibre initiation stage affects fibre development (Ruan *et al.*, 2003; Ahmed *et al.*, 2019). During secondary cell wall synthesis, SuS channelises UDPG to cellulose synthase (CES) located in cell membranes of fibres. Toward the end of the elongation phase, cellulose synthesis is hastened and leads to secondary wall deposition (Haigler *et al.*, 2001). The high SuS activity at different stages of fibre development has a potential towards sucrolysis of sucrose into component hexoses, which are used for cell elongation. The increased cellulose contents will result in improved fibre smoothness and strength.

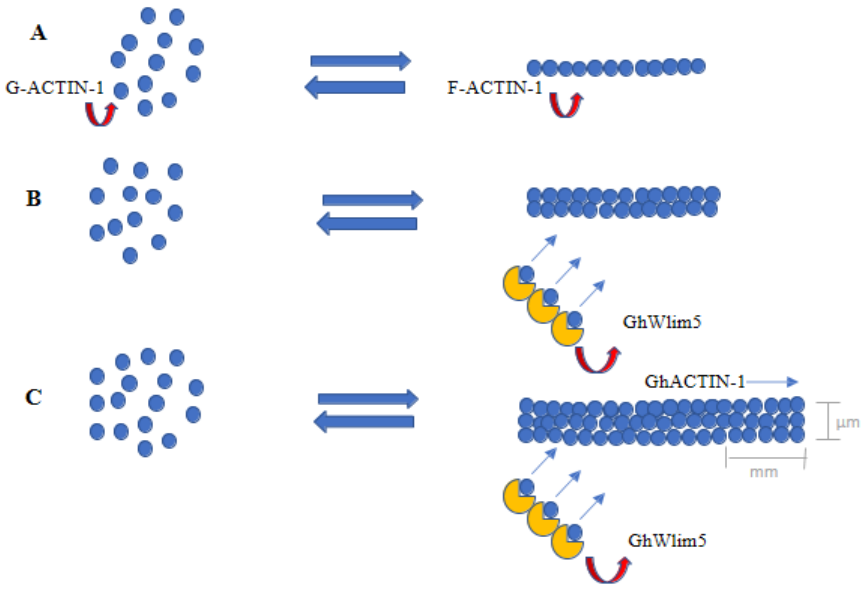

Figure 2: Schematic diagram of actin filament elongation and bundling by *GhACTIN-1* and *GhWlim5* in cotton fibre
(A) Cotton fibre elongation by *GhACTIN-1* gene when expressed under normal condition (B) Actin filaments bundling by *GhWlim5* gene when expressed under normal condition (C) Actin filament elongation and bundling by over-expression of *GhACTIN-1* and *GhWlim5* genes resulted in increased cotton fibre length and strength respectively (Iqbal *et al.*, 2019).

Effect of Cellulose Content on Fibre Development

The process of cellulose synthesis is catalysed by the enzyme cellulose synthase (Saxena *et al.*, 1994). A number of bacteria such as *Agrobacterium, Azotobacter, Pseudomonas and Acetobacter* are naturally found to be pronounced cellulose producers (Wong *et al.*, 1990). Among these the most efficient cellulose synthesising bacteria come from the genus *Acetobacter,* especially *Acetobacterxylinum* which was later on reclassified as *Gluconacetobacterxylinus* by Yamada *et al.* (1997). Bacterial cellulose (BC) is known to possess several unique characteristics in comparison to plant celluloses such as ultra-fine network of cellulose microfibrils, increased water holding capacity, high strength and high moldability (Nishi *et al.*, 1990). Hence, transformation of the Bacterial cellulose synthase (*Bcs*) genes in cotton can play a significant role in improving the cotton fibre quality. In *G. xylinus* strains the process of cellulose synthesis was found to be regulated by an operon consisting of four genes, *acsA, acsB, acsC* and *acsD*. Out of these, *acsA* and *acsB*, were reported to be essential for the production of cellulose in-vitro (Lu *et al.*, 2002).

Role of Flavonoids Pathway on Cotton Fibre Improvement

Flavonoids, along with various pigments and co-pigments, contribute to different flower colours (Grotewold, 2006). The role of flavonoid pathway in cotton colour development and its potential in improvement of fibre quality was reported by Liu *et al.*, (2018). Therefore, it is possible that fibre quality parameters can be enhanced through over expression of flavonoid genes (Ahad *et al.*, 2018).

Marker-assisted Selection for Fibre Quality Improvement

The majority of fibre properties are under genetic control and are inherited quantitatively. For example, the length, strength and fineness of cotton fibre are influenced by 12 to 21 Quantitative Trait Loci (QTLs) (Park *et al.*, 2005). Significant improvement in fibre length and fibre strength (~70%) can be achieved by transgene exploitation through breeding but the later remain unstable in inheritance (May *et al.*, 2002). Until now, 28 different QTLs for fibre quality have been identified (Zhang *et al.*, 2011).

Effects of Phytohormones on Fibre Development

Plants use different hormones to regulate different stages of fibre development (Hao et al., 2012; Tan et al., 2012). Auxin and gibberellins in combination enhanced the fibre growth (Ji *et al.*, 2003, Seagull *et al.*, 2004). Indole acetic acid and phenyl acetic acid improve the fibre length in some cultivars (Gokani & Thaker, 2002). Some hormones like ABA (abscisic acid) have a negative impact on fibre growth (Haigler *et al.*, 2012).

Management of Cotton Fibre Quality through Combined Approach

Fibre quality traits are controlled by several genes. Because of the polygenic control of fibre quality, it is difficult to achieve all the desired characteristics of fibre quality through a single approach, such as plant breeding and or the introduction of a few fibre trait-related genes through transgenic modification — or even through knockout of a few genes responsible for inhibition of fibre related gene expression (Sawant *et al.*, 2018). Expression of individual fibre traits in cotton could lead to improvement in a step-wise manner, thereafter the scope of which can be increased through gene pyramiding of different transgenic traits into a single plant variety through conventional plant breeding combined with knockout of inhibitors in-

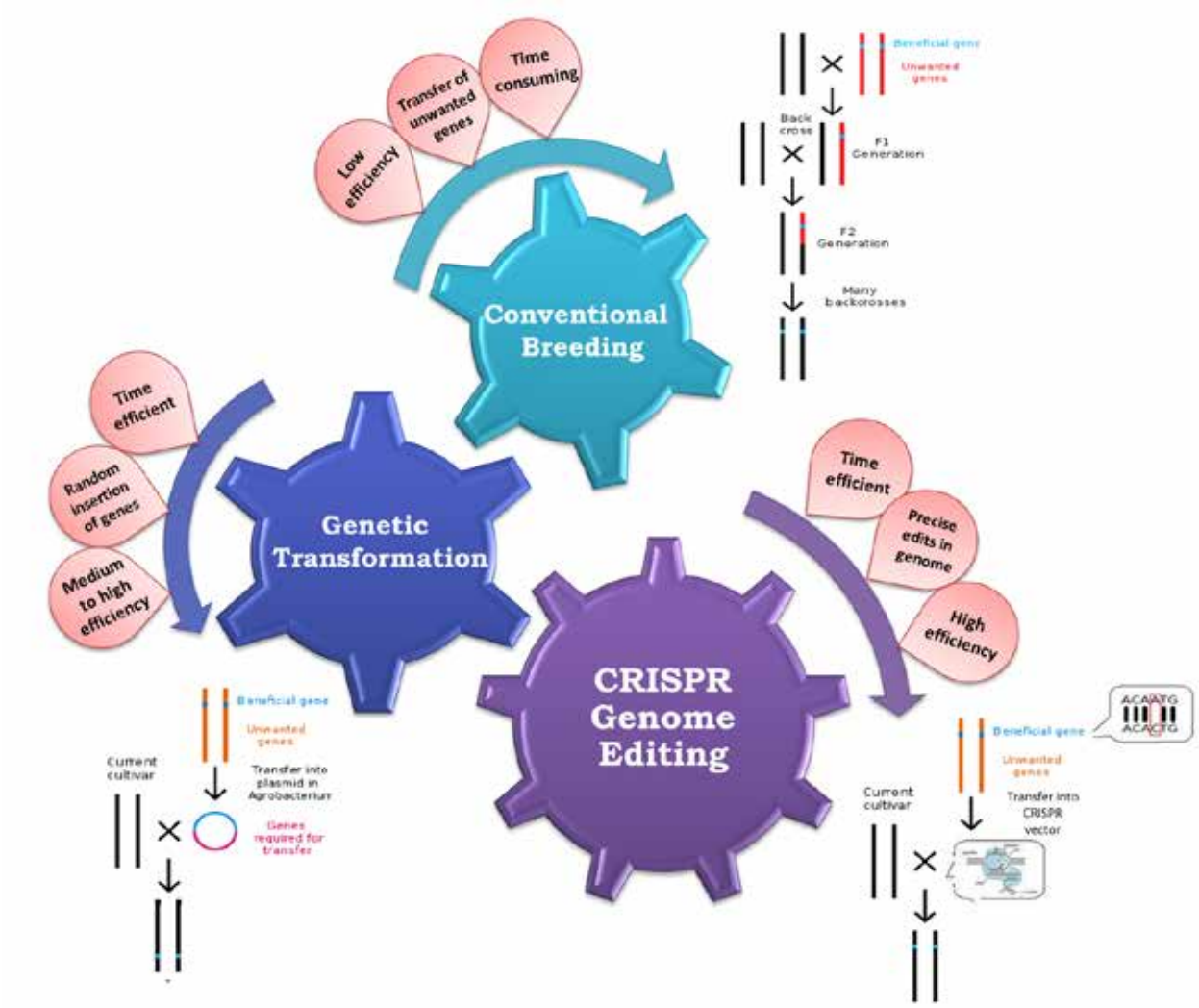

Figure 3: Schematic representation of combinational approach for cotton fibre quality management (Akhtar *et al.*, 2019)

volved through a genome editing technology, like CRISPR/Cas system to develop varieties harbouring all these traits to meet the growing demands of consumers and the textile industry. It will be helpful to achieve these difficult tasks in a short time by improving fibre properties using a combination of approaches that could save economic losses due to poor quality fibre waste that are generally incurred by textile industry (Li *et al.*, Morello *et al.*, 2019).

Conclusion

Improvement of cotton fibre quality is a topic of great interest for scientists and the textile industry. Any efforts to improve fibre traits will lead to greater success for the cotton sector. Scientists are trying to work on various factors that may enhance quality of cotton fibres. With the advent of new technologies, a combined approach to target more than one aspect simultaneously will be more fruitful.

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