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Current and Future Prospects of Plant Breeding with CRISPR/Cas

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Authors' contributions

This work was carried out in collaboration between both authors. Both the authors of this publication contributed equally in reviving the literature and writing this manuscript. Author PMB mainly dealt with CRISPR/Cas discovery and its technological perspectives while author WSG was mainly focused on applications part of the CRISPR/Cas technology in plant breeding. Both authors read and approved the final manuscript.

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ABSTRACT

Innovative plant breeding technology is an absolute necessity to enhance agriculture production in order to have an ambition of feeding nutritious food to the ever-increasing population. Current advances in CRISPR/Cas genome editing technology have led to effective targeted changes in most plants that promise to accelerate crop improvement. Here we discussed the discovery of CRISPR/Cas technology, associated manipulations for plant genome editing and its potential applications in the plant breeding. We emphasized mainly on the most essential applications of CRISPR/Cas genome editing in crop improvement, such as crop trait improvement (yield and

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biotic/abiotic stress tolerance), developments in optimizing gene regulation, strategies for generating virus resistance in plants, and the use of high throughput mutant libraries. Finally, the challenges and opportunities for plant breeding in precision agriculture and its bright future discussed.

Keywords: Genome editing; CRISPR/Cas; precision plant breeding; trait improvement; future plant breeding.

1. INTRODUCTION

In the early 1800s the population of the world was estimated to be around one billion. It grew to two billion in 1920's and to 6 billion at the close of the last millennium. We are currently adding approximately 80 million more per year and, at this rate, the global population will increase from the current 7.5 billion to more than 10 billion by the 2030swith global demand for food rising by 100 to 110 percent compared to 2005 [1]. These are the grand challenges of the 21st century. This must be done in the face of changing consumption patterns, the impacts of climate change and the growing scarcity of water and land. Crop production methods will also have to sustain the environment, preserve natural resources and support livelihoods of farmers and rural populations around the world. There is a pressing need for the 'sustainable intensification' of global agriculture in which yields are increased without adverse environmental impact and within the land available for cultivation. Addressing the worldwide food security requires an urgent international effort with a clear sense of long-term challenges and possibilities.

If there is one feature that distinguishes plant from animal life on our planet, it is the reliance of plants on solar energy to generate molecules with energy-rich bonds, the fuel that will be used by almost the entire biosphere (including plants themselves) to build other organised molecules and drive the rest of the processes that we know as life. Food is the most important primary need in human life and agricultural crops are the main source of staple food. Crops provide food, feed, fuel and other consumer resources as well, to human life and thus make an enormous contribution to society. So, crowd innovation is urgently needed for breeding technologies to boost agricultural production, productivity and to speed up sustainable agricultural development in order to feed and feed a rapidly increasing population. The major crop improvement methods in modern agriculture are cross-breeding, mutation breeding and transgenic breeding. Cross-breeding brings desirable alleles and increases variability through genetic recombination [2]. Because of different centuries of direct reproductive development, large parts of important crop genomes are fixed, and genetic variability has reduced substantially, which reduces the ability to increase many features.

Insights in traditional plant breeding: Plant breeders have a variety of methods at their disposal; from selective cross-breeding to innovative genome editing methods. During the 20th century new plant breeding techniques based on new scientific insights and technological developments were introduced. Genome editing is the latest addition to these breeding techniques. It is important to note the following; regardless of whether these methods have been developed recently or have existed for thousands of years, all plant-breeding techniques affect the plant's DNA. Mutations are the source of genetic variation especially in DNA and typical traditional process involves waiting for spontaneous mutations followed by human selection. Until the beginning of the 20th century, plant breeding was mainly an empirical selection process in which seeds or tubers from the best-adapted crops were stored for the following year. This selective crossbreeding was based on spontaneous DNA mutations that occur in nature. By introducing genetic mutations utilizing chemical mutagens or physical irradiation [3], mutation breeding has widened genetic variation. Processes are limited by their stochastic nature, and it is difficult to create and screen big numbers of mutants. Such time-consuming, laborious and unexpected Breeding programs cannot keep pace with rising crop manufacturing needs, even if marker-assisted breeding strategies are implemented to enhance selection effectiveness [2]. Transgenic breeding can break the roadblock of genetic segregation, which produces desired characteristics by transferring exogenous genes into elite background varieties.

These mutations may be due to errors that occur during the replication of DNA that takes place during cell division or may arise under the influence of radiation from the sun. However, not every change to the DNA sequence leads to new

traits. In most cases, mutations do not result in changes to the phenotypic characteristics of the plant. However, in certain situations, changes in a plant's DNA can result in new beneficial or detrimental characteristics. These changes contribute to genetic variation. Switching off this gene in other plants also gives their flowers a cauliflower-like appearance [3].

Mutation-based plant breeding: The greater the genetic variation within a species, the more opportunities there are to find and combine desirable characteristics. In addition to spontaneous DNA mutations, plant breeders started to use mutation breeding in the 1930s to introduce additional variation and create new crop traits. This type of breeding uses radiation or chemicals to make changes to plant DNA at a high rate [4,5]. This increases the genetic variation available for plant breeding. The result of all this irradiation is a large collection of seeds with different random DNA mutations. These seeds are then used in breeding programs to get rid of the unwanted mutations and to identify plants with desirable, improved characteristics. Traditional mutation breeding has resulted in 3,200 improved crop varieties in more than 175 plant species, including rice, maize, wheat, banana, tomato, pumpkin and soya. The striking color of the flesh and the sweet taste of the pink grapefruit is a good example of a new crop characteristic created by this form of mutation breeding [2].

1.1 What are ZFNs, TALENs and CRISPR/Cas9

Zinc-finger nucleases (ZFN's) include fusion proteins with site-specific DNA binding domains attached to the bacteria's FokI restriction enzyme. Each of these domains can recognize a 3-4 bp DNA sequence, and tandem domains can bind an expanded segment of DNA consisting of multiple sequences of 3 bp such as 9, 12, 15 or 18 bp typical of a cell's genome. ZFNs are designed precisely as a pair to cut flanking regions on both sides of the targeted site. Similar to ZFNs, transcription activator-like effector nucleases (TALENs) are fused artificially to the FokI cleavage domain with a customized array of TALEs. The TALE repeat amino acid sequences are identical with the exception of di-residues at positions 12 and 13, known as repeat variable di-residues (RVDs). In 2013, three autonomous organizations created a rice (*Oryza sativa*), wheat (*Triticum aestivum*), *Nicotiana benthamiana* and *Arabidopsis thaliana* system [6,7,8].

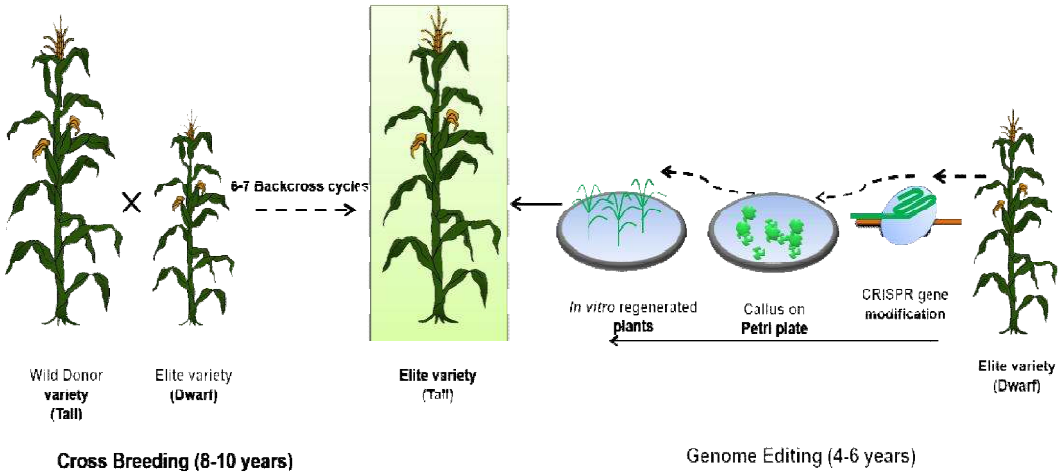

Fig. 1.Effectiveness of plant breeding, when complemented with genome editing compared to the traditional cross breeding method. Traditional cross breeding method requires a period of about 8-10 years for improving a trait (e.g., height) of an elite recipient line from a donor line. This process requires multiple back crossing events with the recipient elite line for several generations to eliminate unexpected linked traits to introduce the desired trait from the donor line. In contrast, plant breeding by genome editing takes only a half time (4-6 years) compared to traditional cross breeding method. This involves improving a trait by precisely modifying the regulatory elements of a target genes or the target gene itself in elite recipient varieties

CRISPR *(clustered regularly interspaced short palindromic repeats) system.* Plant breeders, for the first moment in history, were able to regulate the particular implementation of targeted variation of sequence, which offers a resource to change rapidly for agricultural crop improvements. Continuous improvements have since rendered gene editing a widely accepted, cost-saving, easy-to-use targeted genetic manipulation tool for many plants in CRISPR/Cas systems such as CRISPres1 [9] and nuclear substitution instruments for basic editing [10]. The features changed by genome editing include output, quality and resistance to biotic and abiotic stress. This strategy also has improved hybrid breeding methods and the elimination of undesired characteristics or the addition of requisite characteristics to the elite species is now an easy method that can modify crops properly in just one generation. Therefore, CRISPR/Cas is able to improve global security of food and sustainable farming. CRISPR is a clustered family of short DNA repeats which is in fact is a part of aprokaryotic adaptive immune system (bacteria (40%) and archaea (90%) [11,12].

1.2 Discovery of CRISPER/Cas9

The CRISPR/Cas9 system study started with repetitive DNA segment findings in *E. coli* in 1987 [13], but Barrangou and colleagues confirmed their function later in 2007. They protect bacteria or archaea from invading viruses [14]. These segments correspond to the cellular mechanism of the unicellular bacteria or archaea in the bacteriophage [11,12]. It mainly comprises of two parts, first is regularly interspaced identical short DNA repeats (20-40 bp in length) and they are palindromic in nature. Second part is hypervariable spacer DNA and each segment of spacer DNA is unique and matches up perfectly with the viral DNA especially bacteriophages also called as protospacers [15]. There are also numbers of other associated genes with CRISPR [15] called as *cas* genes. *cas* genes will produce Cas proteins which are either helicases that unwinds DNA or nucleases that cleaves DNA [15]. Based on differences in their components they are of two major classes of crisper systems that is class 1 and class 2 systems. RNA guided target cleavage in class 1 systems (types I, III, and IV) requires a complex of several proteins, while in the class 2 systems (type II, V and VI), only one RNA-guided endonuclease (e.g., Cas9 in type II) is required to mediate cleavage of invading genetic material [16].

When foreign DNA is recognized, the bacteria transcribe and translate proteins (Cas) and it also transcribes a repeat DNA to produce crisper-RNA (crRNAs). At the 5' end, the crRNA contains the spacer, a short segment of RNA that complements a sequence from a foreign genetic element, and the 3' end contains a piece of the CRISPR repeat sequence. This crRNA then combines with Cas proteins which will then degrade the bacteriophage DNA upon hybridization with the crRNA. So, before the infection starts, the infection has essentially ended. crRNA with tracer RNA is responsible for recognizing the base sequence and its cleavage, while Cas leads to further cleavage. A defining feature of CRISPR/Cas systems is the assembly of mature crRNAs with Cas proteins into crRNA–effector complexes to interrogate DNA targets and destroy matching sequences in foreign nucleic acids. Notably, a short-conserved sequence motif (2–5 bp) located in close proximity to the crRNA-targeted sequence on the invading DNA, known as the PAM, plays an essential role in target DNA selection and degradation in most CRISPR/Cas systems. In case where cell don't have a spacer DNA that matches bacteriophage DNA, the cell produces a different class of Cas protein (class 1) which takes the DNA in, breaks it apart but more importantly it takes that DNA and insert a copy of it into the crisper system. So, spacer is actually a history of all old infections so that cell won't be infected again [17-19].

1.3 Insight in CRISPER/Cas9 Technology

The system includes CRISPR repeat-spacer arrays and Cas-proteins. CRISPR/Cas systems are classified into two classes, which have been further divided into six types based on their *cas* genes signature, based on their *cas* genes and the nature of their interference complex. The Class 1 system of CRISPR/Cas uses interference multi-cases of proteins, while the systems of Class 2 do interfere but with complex single-effector C protein. The CRISPR system for genome modification is based on RNA-led DNA interference [20]. Jennifer Doudna and Emmanuelle Charpentier used this mechanism to create current crisper system which is a powerful RNA-guided DNA targeting platform for genome editing. The Cas9 protein (endonuclease) of bacterial immune systems, is emerging as a powerful tool for engineering the genome in diverse organisms as Cas9 system (Type II of class 2 system) requires only one protein and it is easy to manipulate compared to the proteins

(class 1 system) that requires multiple components to act. As an RNA-guided DNA endonuclease, Cas9 can be easily programmed to target new sites by altering its guide RNA sequence, and its development as a tool has made sequence-specific gene editing easier. Basically, sgRNA comprises of tracrRNA (trans-activating crRNA) responsible for attaching to Cas9 and crRNA responsible for specific target binding [21-23].

Two steps are involved in the Cas9-mediated genome editing process: first one is DNA cleavage which is followed by DNA repair. Cas9 (in PAM's presence), directed to a specific genomic locus under guide RNA (sg RNA) direction creates a double stranded break which triggers cellular repair mechanisms like non-homologous end joining or homology-directed repair [25]. In non-homologous end joining, a random insertion or deletion mutation at the double strand break site may lead to gene knockout caused due to change in the reading frame of the gene or due to a mutation in the critical region of the encoded protein. While in case of homology-directed repair, it can be used to generate a desired sequence modification or sequence replacement at the double stranded break site through homologous recombination guided by a donor DNA template, causing targeted gene deletion, mutagenesis, insertion, or gene correction [26-28]. Thus, the CRISPR/Cas9 system provides a powerful platform for sequence-specific genome editing, including gene knockout, gene knocking, and site-specific sequence mutagenesis and corrections. The first system showing a specific cleavage of DNA *in vitro* and in eukaryote [29-32], was Type II CRISPR / Cas9 from *Streptococcus pyogenes*. Type II CRISPR/Cas9 system has been repurposed with two components: The Cas9 nuclease and one RNA guide (sgRNA), which comprises a crRNA fusion and a transactivating crRNA. The CRISPR/Cas9 system now has two components and a single RNA guide.

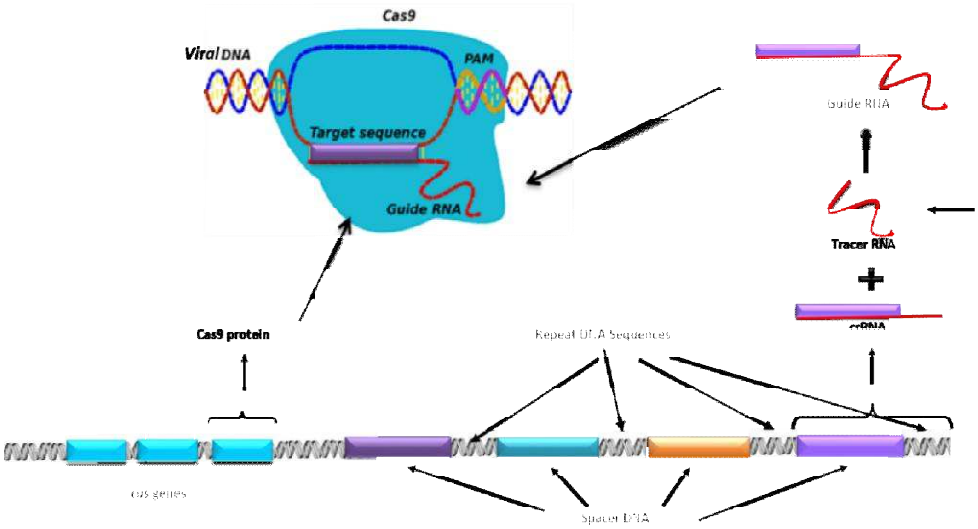

Fig. 2. CRISPR/Cas9 system in bacteria:Insertion of viral DNA into the bacterial cell upon viral infection triggers the crisper/Cas9 system. Upon novel phage infection, a new spacer derived from the inserted genetic material is incorporated into the CRISPR array. Reinfection by same virus leads to transcription of spacers with repeats to produce crRNA. The tracrRNA is transcribed separately which then anneals to the crRNA to produce guide RNA. Role of tracrRNA is to hold crRNA in position in cas9 assembly where hybridization of target DNA with guide RNA especially crRNA then leads to conformational change in Cas9 which then cleaves the double stranded target DNA preceding the PAM sequence by its endonuclease activity. This immune system of bacteria has been modified by the scientist as a powerful RNA-guided DNA targeting platform for genome editing which can be used either to inactivate a gene or embed a new gene into the bacterial, plant or animal cells. This technology allows one to precisely manipulate virtually any genomic sequence specified by a short stretch of guide RNA [24,132]

2. PLANT BREEDING APPLICATIONS OF CRISPR/Cas

The breeding process is generally composed of six steps for CRISPR/Cas approach. Take, for instance, wheat. We are very vulnerable to mildew, a fungal disease, in wheat species that we grow today. CRISPR/Cas has now been used by the researchers to develop a type of mildew resistant wheat. The following 6 steps used in this approach are: A detailed genome study is always preceded by a successful result of CRISPR/Cas-based breeding. The crop feature you want to modify (in this example, mildew sensitivity) must first be thoroughly analyzed at the genetic and molecular level. Upon determining which changes in DNA are necessary to increase the fungal resistance of the plant, scientists design a CRISPR-RNA molecule. This RNA molecule determines the exact location, wherechanges in DNA sequence is required. In the plant cell, either by transforming agrobacterium, plant viruses, or directly as a protein RNA complex, DNA-cleaving enzymes and guiding molecules of CRISPR RNA should be introduced [30,31]. In these two cases, there is no genetic material integrated into the plant DNA. Casand CRISPR are spontaneously dis-assembled by the plant cell after their editing tasks are completed. The result is that the target gene(s) have the requiredmutation(s). This resulting plant cannot be distinguished from the one that spontaneously or through traditional mutation breeding has acquired mutations. The next step is to track the cells and/or tissue parts in which the desired change (or changes) are effected correctly by the CRISPR/Cas system. This is often done by using DNA sequencing to see if the approach succeeds. A complete plant is then grown from modified cells or plant tissue cultures. In the final stage, traditional methods of plant breeding incorporates the desired mutationin to the elite varieties. Genome editing in plants genome editing techniques like CRISPR/Cas has mainly been used in plants to induce highly controlled and accurate DNA mutations. We're talking about precision breeding', therefore a gene can be switched off or on in the plant by effectively achieving a targeted DNA change. This makes it possible for breeders to dampen and/or strengthen unwanted characteristics. These mutations may also occur in nature spontaneously. Genome editing has the advantage that only desired mutations are produced without further unwanted mutations.

2.1 Improvement of the Crop Trait

The elimination of negative factors is an excellent genetic improvement strategy. The easiest and the most common application is CRISPR/Cas9 to knockout genes with unwanted characteristics (Fig. 3B). This approach also enhanced hybrid cultivation techniques and many other important aspects of crop productivity [32].

2.1.1 Crop yield improvement strategies

Genetic modification is the principal aim of crop improvement, due to the need to improve food safety. Rendering is a complex characteristic based on numerous factors. The CRISPR/Cas9 is shown to be an effective technology for the improvements of yield-related effects in plants with loss of function mutations, which results in negative regulators known to affect grain number (*OsGn 1a*) or the weight of kernel grain (*OsGS3*) (*TaGW2, OsGW5, OsGLW2* or *TaGASR7*) as well as panicle size (*OsDEP1*, *TaDEP1*) and the number of tiller (OsAAASR7), pathogen resistance (OsRDR6), of plants. The quality characteristics revealed are different depending on the specific reproductive requirements. To date, improvements in quality through genome editing have affected the content of starch, fragrance, nutritional value and storage quality of crops. The knockout of Waxy through CRISPR/Cas9 [33,34] has generated rice with low amylose content and thus an improved diet and cooking quality. The CRISPR/Cas9 knockout waxy maize lines produced by DuPont Pioneer have high commercial use rates [35]. CRISPR/Cas9 has also been used to produce stubborn high amylose rice by mutating *SBEIIb*, the branching starch enzyme, which is supposed to benefit chronically related patients with high-amylose food [36]. Fragrance is an important rice quality feature and has increased commercial value with desired aromas. A defect in betaine aldehyde dehydrogenase 2 gene in a biosynthesis of 2-acetyl-1-pyrroline (BADH2),is the major fragrant for fragrant rice. An aromatic rice line is established using TALEN-targeted *OsBADH2*disruption, which is similar to the natural mutant fragrant rice variety [37] with a similar level of 2-acetyl-1-pyrroline (0.35–0.75 mg/kg). Gluten proteins from cereal crops trigger celiac disease in more than 7% of individuals in western countries. The α-gliadin gene family, the major gluten-encoding gene family in wheat, consists of nearly 100 genes or pseudogenes. CRISPR/Cas9 editing offers a new way to alter

traits controlled by large gene families with redundant functions. Indeed, by simultaneously knocking out most conserved domains of α-gliadin family members, researchers have created low-gluten wheat [38].Other high-quality crops produced by CRISPR/Cas9 editing include seeds with high oleic acid oil in *Camelina sativa* [39,40], and *Brassica napus* [41], tomatoes with a long shelf life [42,43], high-value tomato with enhanced lycopene [44]or γ-aminobutyric acid content [45,46], and potato (hairy roots) with reduced levels of toxic steroidal glycoalkaloids [47].

2.1.2 Developing stress resistance in plants

The main factors that influence crop yield and quality are stresses. CRISPR/Cas9 knockout have been able to obtain many plants with increased biotic stress resistance, including fungal, bacterial,viral diseases and insects. Powdery mildew, for example, in crops is a devastating fungal disease. But with help of TALEN and CRISPR/Cas9, now plants with an increased powdery mildew resistance available [48]. Likewise, Nekrasov et al. [49] have shown that MLO mediated CRISPR/Cas9 confer resistance to powdery mildew in tomato. Another example of fungal destructive disease is the rice blast where the gene of ethylene responsive transcription factor (*OsERF922*) [50] Was knocked out to confer resistance to rice. With regard to viral diseases, CRISPR/Cas9 has also produced tungro disease-resistant rice [51], broad potyvirus-resistant cucumber and curling cotton leaf disease-resistant cotton [52]. Recent report shows the *OsCYP71A1* interruption of serotonin biosynthesis and significantly increased concentrations of salicylic acid conferred resistance to the two most destructive rice pests by crops and stem borers. Contamination of arable land among abiotic stresses has created the need to prevent crop toxic heavy metals from accumulating. Rice varieties with low concentration of cadmium, radiocesium, and arsenic have been developed by breeder *OsARM1*, *OsNramp5* and *OsHAK1* [53-55].

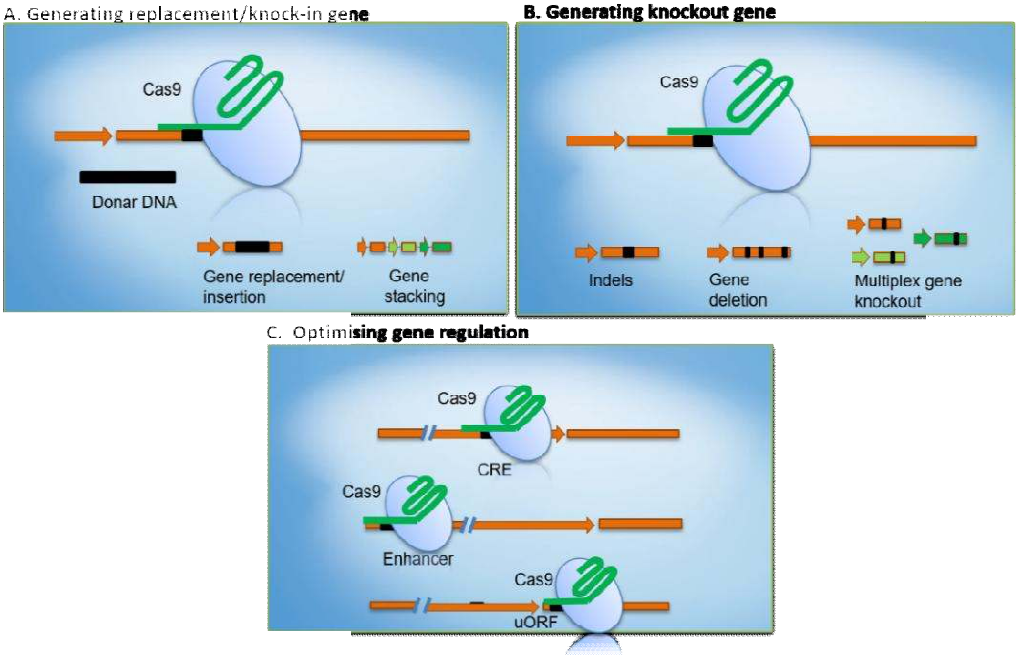

Fig. 3. Potential applications of CRISPR/Cas technology in plant breeding. (A) Gene replacement and knock in gene insertion achieved either by non-homologous end joining or by homology-directed repair can help in achieving gene stacking, gene editing for gain-of-function to obtained new traits in recipient elite lines. (B) Mutations created with the help of this technology can achieve indels, gene deletions, and multiplex gene knockout. (C) It can also be used to control the expression of the target gene by editing the regulatory sequences of that gene such as promoter or enhancer region. Abbreviations: CRE, cis-regulatory element; uORF, upstream open reading frame

2.1.3 Plant breeding on fast tract

Hybrid breeding is a powerful way to boost crop productivity. A male-sterile maternal line is a precondition for producing a high-quality hybrid variety. Superior advances have been made in the use of CRISPR/Cas-mediated gene knockout to produce male sterile lines in rice [56] and maize [57,58], male-sterile photosensitive genes in rice [59] and *ms45* [60]. The main hindrance to the use of heterosis in breeding is Hybrid Sterility. *SaF/SaM* in the sterility locus *Sa* and *OgTPR1* in the locus *S1* [61], were disrupted in order to overcome the reproductive barriers in the *japonica-indica* hybrids. The knockout of one or other Shen et al. [62], found The knockout in the indica allele *Sc-I* of one or two copies of the *Sc* gene has found that it has, in *Japanese-indica* hybrids, also rescued the male fertility. Similarly, The increase in fertility of the *Japonica-indica* hybrids in the knockout of the *ORF2* toxin genes, responsible for the newly found selfish-gene suicide mechanism in rice [63]. The asexual propagation lines were established, both through simultaneous activation of *BBM1* in Egg cells [64,65] and through the knocking out of *MTL* [66], which allowed the heterozygosity of hybrids to be fixed by the propagation of seeds. Genome revision is also an effective approach that helps to increase the resistance of silicon shattering [66] and to overcome auto-incompatibility of diploid potato [67], for example by enhancing haploid breeding [68,69], reducing growth time [70], increasing siliconic shatter resistance.

2.2 Crop Trait Improvement via Replacement and Knock-In Gene

Many agronomic traits are conferred by single-nucleotide substitutions, gene expression changes, or the addition of new gene functions. Precise gene modifications such as knock-ins and replacements facilitate breeding by introducing new alleles without linkage drag or generating allelic variants that do not exist naturally [71]. Moreover, knock-in can be used to alter multiple elite traits by stacking genes in a single variety. Therefore, knock-ins and replacements have great value for crop trait improvement (Fig. 3A). Unfortunately, because HDR is an infrequent DNA repair pathway, these techniques are far from routine, and their use in trait improvement has thus far been quite limited. Nevertheless, CRISPR/Cas9-mediated gene editing to improve drought tolerance in maize. The edited ARGOS8 variants had elevated ARGOS8 transcript levels and increased yields under drought stress [72]. Also a tomato line created with long shelf life by editing a T317A replacement into the *ALC* gene [73]. For HDR efficiency, a geminivirus-based DNA replicon has been used to increase the number of repair templates, which increased gene-targeting efficiency in potato [74], tomato [75-77], rice [77], wheat [78], and cassava [79]. For example, using geminivirus replicons, tenfold increase in the frequency of insertion of the cauliflower mosaic virus 35S promoter upstream of *ANT1* in tomato; constitutive expression of *ANT1* led to the generation of a purple tomato with increased anthocyanin content [80]. Substitution of key amino acids in the conserved domains of *ALS* and *EPSPS* can confer resistance to sulfonylurea-based herbicides or glyphosate. Sulfonylurea based herbicide-resistant soybean [81], maize [82,83], and rice [84-88], plants were generated through HDR-introduced nucleotide changes in ALS. Similarly, *T112I/P116S* (TIPS) and T102I/P106A double amino acid substitutions of *EPSPS* were introduced into flax (*Linum usitatissimum*) [88] and cassava [88] by selecting for HDR-generated glyphosate resistance. Owing to the low rate of HDR, efforts were made to create *TIPS EPSPS* glyphosate-resistant rice via an intron-targeting strategy involving NHEJ-mediated gene replacement and insertion [89]. Although indels may arise at the junctions of the recombination sites of the targeted intron, the final gene transcript is not affected. This method represents a higher frequency alternative to HDR-mediated gene targeting in plants.

2.3 Optimization of Gene Regulation in Plants

In order to test genetic function and to greatly facilitate plant breeding, the modulation of gene expression is not just the formation of mutations in coding sequences. Gene expression can be affected at various levels, including transcription, mRNA and mRNA processing. These processes are controlled by a set of cis-regulatory elements that can be modified by modifying the genome (Fig. 3C). To date, the focus of plants genome editing is on promoters such as substitutes and the removal of cis-regulatory elements [98-102]. The quantitative characteristic region promoters of genes like *SlCLV3*, *SlS* and *SlSP* have been edited, creating a continuum of variations and selecting mutated allels with improving metrics. Translation upstream open reading frames (uORFs) may also be used for gene control, which are well-known cis elements

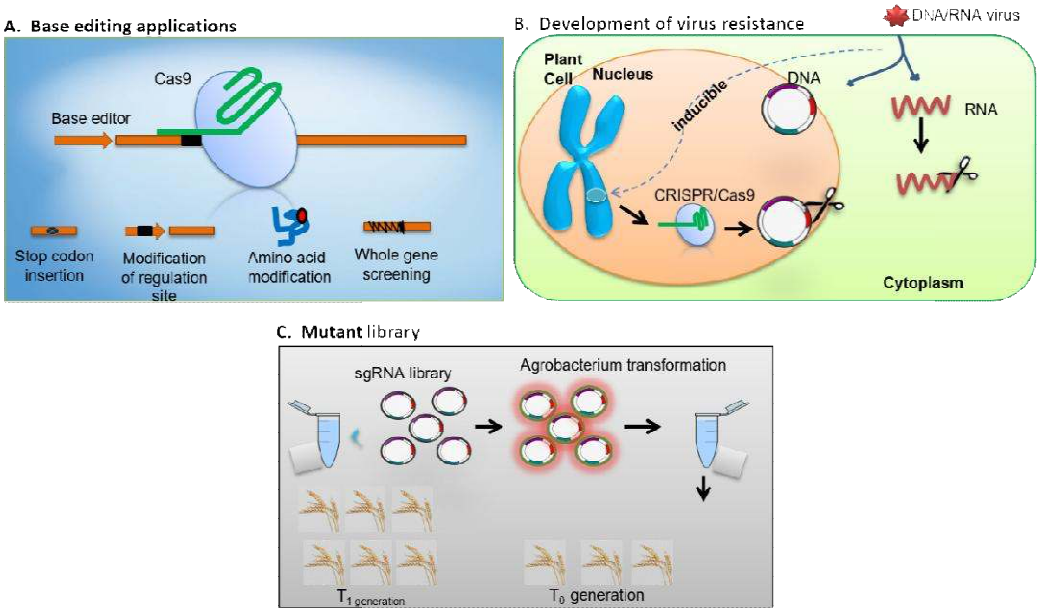

Fig. 4. Few more potential applications of CRISPR/Cas technology in plant breeding. (A) Base editing can have many applications in crop trait improvement, like amino acid substitution, stop codon insertion in order to disrupt the gene function and gene regulation. (B) CRISPR/Cas technology can be used to create virus resistant plants by integrating guide RNA targeting DNA or RNA viruses into the plant genome. (C) CRISPR/Cas-based genome-wide screening, is a valuable technique for functional genomics and genetic improvement. Abbreviations: sgRNA, single guide RNA

that often have adverse effects on translation and mRNA decline that are unsensed. Bioinformatics analysis forecasts UORFs among plant mRNAs. Regulating DNAase 1 hypersensitive web sites is also an efficient way of finding supposed enhancer elements [103-110], and this is a good means of identifying them.

2.4 Applications of Base Editors in Plants

In either coding or non-coding regions, several agriculturally important characteristics are conferred by one-nucleotide polymorphisms. Base editing is very helpful for plant breeding and crop improvement.The conferment of herbicide resistance is an important use of basic editing in coding areas (Fig. 4A). AALS was targeted at plant-cytidine base editor and haloxyfop-R-methyl rice was produced by targeting acetyl-coenzyme *Acarboxylase ACCase* gene with a plant adenine base editor [90]. The rice was created using a sulphonylurea or imidazolinone resistant rice [91], wheat [92], arabidopsis [93,94] and watermelon. Alternative splicing is a regulatory process of gene expression that results in a single gene encoding multiple proteins, which can greatly increase the diversity of proteins capable of producing new traits. Base editing is also used to regulate RNA splicing pathways. Base editing can generate point mutations at these conserved nucleotides, leading to the loss of particular splice forms or mis-splicing [95]. In addition, Li et al. [97] created mRNA mis-splicing-induced null mutants of *AtMTA* and double mutants of *OsGL1* and *OsNAL1* in riceG to A in the splice donor site, resulting in the constitutive retention of an intron of *AtHAB* and hypersensitivity to abscisic acid [96].

2.5 Plant Breeding Strategies for Antiviral Resistance

Around half of all vegetable diseases are thought to be caused by viruses that lead to massive losses in worldwide agricultural production [112]. Because the CRISPR/Cas system provides an archaea and bacteria-invading defense mechanism that cleaves plasmids, DNA viruses and RNA virus, it can also be used for plant viral resistance (Fig. 4B). Geminiviruses are DNA viruses on the single-stranded rotational ring with a dual-stranded intermediate. Stable Cas9 and

sgRNAs overexpressed specifically to the geminivirus genomes to prevent their replication were used for antiviral breeding in plants [113-115]. However, NHEJ pathways indelsare developed at DSB sites to generate viruses that are capable of exempting from Cas9/gRNA cleavage [116]. As an intergenic stem-loop sequence is essential to the replication of geminiviruses and intergenic sequences where indel typically lose replication activity, these sequences are ideal targets for the development of geminivirus-resistant plants [117]. A drawback of the CRISPR/Cas system is that Cas9/sgRNA is a constituent expression that is suited to cause off target changes but can reduce off-target effects to an undetectable level using a virus promoter to drive Cas9 expression [118]. RNA viruses cause more losses in farm production compared to DNA viruses [119]. FnCas9 binds PAM-independently for the RNA, inhibiting hepatitis C virus in mammalian cells from translation and replication [111]. In addition, the replication of cucumber mosaic and the tobacco mosaic virus in plants is efficiently suppressed by FnCas9 [111]. C2c2 may split single-seam RNA (2) and interfere with plant replication of turnip mosaic viruses [112], in contrast to most Cas proteins.

2.6 Plant Mutant Libraries

Mutant libraries with whole-genome-scale are valuable tools for the improvement of genomic functions and genetics (Fig. 4C). Traditional mutant libraries are based on random mutations of substances such as irradiation, insertion of T-DNA, mutagenesis and transposons of ethyl methanesulfonate (EMS). However, it takes a number of generations to stabilize mutant loss of function, and it takes a long time to determine the relationship between phenotype and genotype between mutants. Two large groups of the knockout mutant library generated by CRISPR/Cas9 covered most of the genes of rice [113,114]. Meng et al. targeted nearly 13,000 rice shooting genes, with more than 14,000 independent T_0 lines. Finally, a target of almost 13,000 genes highly expressed in base tissue rice-shooting and obtaining more than 14,000 separate T_0 lines Produced immunity-associated mutant libraries of leucine-rich recurring genes in subfamily XII, including 54 tomato members. The availability of high quality, uniformly distributed high-coverage knockout mutant libraries could help to develop innovative germplasm strategies and to improve crop features.

3. FUTURE PROSPECTS

Potential in Domestication of Wild Plants and Plant Synthetic Biology: For thousands of years, modern plants have been selectively produced and important properties have been introduced to enable mechanical harvesting of high-quality foodstuffs rich in nutrients. This process has however, led to a loss of diversity that, in certain environmental conditions, can affect fitness [116]. The main events for domestication are related to mutations in so-called domestication genes that have significant effects on major phenotypes such as the barley *vrs1* that is responsible for naked-kernel spike number [117] of maize *tga1*; rice *Sh4, Rc, PROG1* and *LABA1* for white pericarp, erect growth, and barbless awn [118]. Virus resitance can be develop against recetenly sequenced viruses of rice such as Rice Necrosis Mosaic Virus [119]. Given the growing number of sequenced plant species, genome editing provides an efficient approach to plant domestication, and thus to expand crop diversity and to increase agriculture sustainability. For example, CRISPR/Cas-enabled domestication events could lead to the production of new plants and a variety of germplasms for breeding in wild or semi domesticated plants. This technology has so far been used to manipulate monogenes related to domestication in wild related plants with polygenic interest characteristics. The winter pennycress (*Thlaspi arvense* L*., Brassicaceae*) is the most attractive target for rapid domestication. Pennycress has a short season, extreme cool tolerance, high productivity of seed oil and distinct covers of crop attributes compared to many other olive plants and is related to other advanced moutar members [120]. The development of the elite domesticated pennycress varieties is greatly supported by gene editing technologies to modify genes that control seed dormancy (*DOG1*), olive quality (*FAE1* and *FAE2*), glucosinolate accumulation (*HAG1* and *GTR2*), and oil content (*DGAT* genes).Tomato is another instance of genome-editing cultivation domestication. Intensive inbreeding cycles are the cause of an increasing amount of biotic and abiotic stress on modern tomato cultivars. Naturally stress-tolerant wild tomato plants can serve as the ideal material for de novo domestication through the precise development of domestication genes. This strategy to accelerate wild tomato domestications for habit, flora, and fruit production and nutritional characteristics has been implemented in two independent studies very recently, with no loss of

wild germplasm stress toling [121]. In fact, the strategy was implemented in only a few years. One group produced plants with higher yields and larger fruits [122]. A wild relative of the tomato was called "ground cherry." In future, new domesticated plants will support agricultural diversity and help resolve many of the challenges associated with sustainable agriculture, with greater tolerance of a wide range of challenging environments including deserts, maritime regions, low-nutrients soils and cold climates. Synthetic biology of plants is an emerging area combining plant biology with the principle of engineering to create new devices with predictable behaviors [123]. It will play an important role in the improvement of traditional crops and enable novel bio production processes to be developed [124]. Plants are the world's major sources of primary metabolites (i.e., proteins, fatty acids and carbonates) and produce a diverse range of valuable medicinal and industrial secondary metabolites. The first transgenic plant, which began the age of plants with new functions, was produced more than 30 years ago. Marking the beginning of the age of designing plants with novel functions. The CRISPR/Cas system has great potential for improving plant design and synthetic biology. Artificial DNA sequences, such as promoters, genes, transcriptional regulatory elements and genome assemblies, can be inserted into plant genomes to modify the behavior of the cell or plant to create new features. Nitrogen is a key element for the development of crops. Most of (*nif*) genes were characterized by nitrogen fastening and relative level of expression [125]. The CRISPR/Cas system could be used to transfer from legumes to cereals like wheat the genetic elements on the Nod factor-signaling pathway to enable the cereal to re-fix atmospheric acid to reduce our dependence on inorganic fertilizers. Moreover, synthetic biology's main objective is to create regulative circuits for plant behavior, which produce new characteristics that enhance crop productivity [126]. The multiplexing of the gene, repression and editing of Cas9-mediated genes offers unparalleled opportunities for designing synthesized transcription factors that can be utilized to build increasingly complex, programmable and efficient gene circuits. Chloroplasts research is another ray of hope to feed this ever-increasing population. Chloroplasts, a semi-autonomous organelle is the site of a wonder process called photosynthesis. Much of the basic knowledge regarding the chloroplasts is still unclear and it need to be explored soon by considering the importance of chloroplast which feeds most of the living organisms on the earth by producing the food through photosynthesis. Many efforts are still ongoing in this regards in different corners of the world [127-130]. In the C4 rice project, for instance, C4 photosynthesis pathway is installed in the rice, difficulties are guaranteed to fix carbon efficiently; so fine tuning gene expression is required in a C4 pathway to optimize protein contents to improve carbon fixation efficiency [131] and we are certainly sure CRISPR/Cas will play an important role in this process in near future.

4. CONCLUSION

With the ambition of feeding ever increasing population in mind, very fast improvements in the agricultural productivity is an absolute necessity. It takes reasonable amount of time for doing this if one follows traditional ways of crop improvements. However, now with the invent of CRISR/Cas, genome editing becomes plug and play kind of approach for the researchers. CRISPR/Cas systems are easy, versatile and robust to create a powerful tool in genetic improvement through gene knocking-out, knock-in, substitution, point mutations, gene control fineness and other modification at all gene loci. Considering together the recent advances in the genome editing technologies especially CRISR/Cas, next generation sequencing, developments in synthetic biology/systems and functional genomics, it will allow enhanced productivity both in terms of quality and quantity in most of the agricultural crops. Finally, only important thing, which need to be coordinated precisely, is the transfer of this technology from lab to the field that requires a quick discovery of important genetic foundations.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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