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450 *Current Genomics*, 2021, *22*, 450-467

REVIEW ARTICLE

Raising Climate-Resilient Crops: Journey From the Conventional Breeding to New Breeding Approaches

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ARTICLE HISTORY

Received: September 16, 2020
Revised: June 29, 2021
Accepted: August 04, 2021

DOI:
10.2174/1389202922666210928151247

Abstract: ***Background*:** In order to meet the demands of the ever-increasing human population, it has become necessary to raise climate-resilient crops. Plant breeding, which involves crossing and selecting superior gene pools, has contributed tremendously towards achieving this goal during the past few decades. The relatively newer methods of crop improvement based on genetic engineering are relatively simple, and targets can be achieved in an expeditious manner. More recently emerged genome editing technique using CRISPR has raised strong hopes among plant scientists for precise integration of valuable traits and removal of undesirable ones.

***Conclusion*:** Genome editing using Site-Specific Nucleases (SSNs) is a good alternative to the plant breeding and genetic engineering approaches as it can modify the genomes specifically and precisely at the target site in the host genome. Another added advantage of the genome editing approach is the simpler biosafety regulations that have been adopted by many countries for commercialization of the products thus generated. This review provides a critical assessment of the available methods for improving the stress tolerance in crop plants. Special emphasis has been given on genome editing approach in light of the diversity of tools, which are being discovered on an everyday basis and the practical applications of the same. This information will serve as a beginner's guide to initiate the crop improvement programs as well as giving technical insight to the expert to plan the research strategically to tackle even multigenic traits in crop plants.

Keywords: CRISPR, genetic engineering, genome editing, plant breeding, stress tolerance, yield.

1. INTRODUCTION

Changing climatic conditions, such as fluctuations in temperature, unpredictable rainfall patterns, changing composition of gases in the atmosphere, *etc.*, has led to a change in land use pattern, change in the microbiota of soil, changes in water balance, *etc.* [1-3] Plants respond to these changing environmental conditions through various mechanisms, such as altered flowering and fruiting time, modification of floral rewards, growth arrest or acceleration. However, all these adaptive measures result in a significant reduction in the productivity of the plants. The effect of adverse climatic conditions is especially pronounced in crop plants, most of which have been bred for increased yield and have a reduced capacity for tolerating environmental constraints [4, 5]

Many studies have indicated that weathering parameters have a strong impact (67%) on the cropping season as compared to other factors like soil and nutrient management (33%). Increasing mean global temperature, which is one of the greatest concerns of mankind, can affect our major food crops like rice, wheat, maize, and soybean, which form the staple diet for two-third of the world's population. It has been reported that even one degree-Celsius rise in global mean temperature can reduce the yields of rice by 3.2%, wheat by 6.0%, maize by 7.4%, and soybean by 3.1% [1].

Climate change has also exacerbated the problem of abiotic stresses like drought and salinity and can also lead to an increase in the number of pests and pathogens by influencing their habitat range, thereby causing a change in the interaction between pests, their natural enemies and the host plant [2-5]. These factors, along with an exponential rise in world's population, are a threat to global food security. It is, thus, necessary to make crops that can adapt themselves to changing climatic conditions and are high yielding, resistant to various diseases as well as tolerant to a multitude of stresses. Thus, to ensure our food security goals, agricultural yields should not just keep pace with the demands of the increasing population but should also be improved under environmental constraints through the development and utilization of stress-tolerant crops. However, due to the low heritability of characters under selection [6], the task of raising high-yielding crops under environmental constraints has been a difficult one. Because of this reason, the scientific community is trying to identify more traits of importance that can confer tolerance to various abiotic stresses.

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1389-2029/21 $65.00+.00

Human beings have been manipulating plants since time immemorial for improved crop quality and production. It started with the selection of plants with 'superior phenotypes' among naturally existing diversity and breeding them to produce better quality hybrids. However, classical plant breeding methods are time-consuming and costly because they involve screening and selection of desirable traits over generations. In order to speed up the development of useful traits in crop plants, scientists started using mutation breeding for obtaining useful variations [7]. More recently, the development of genetic engineering has resulted in the generation of transgenic plants specifically designed to contain unique traits. Improvements in sequencing platforms and annotation technologies have led to the development of advanced reverse genetics approaches like CRISPR/Cas, which is the latest genome editing tool.

Genome editing using Site/Sequence-Specific Nucleases (SSNs) is the targeted manipulation of a region in the genome for the desirable outcomes. Artificially engineered SSNs are a breakthrough technology for creating plants with desirable phenotypes by generating Double-Stranded Breaks (DSBs) at the target sites. These DSBs will get repaired by the cell's in-house repairing pathways *via* Non-Homologous End Joining (NHEJ) or Homologous Recombination (HR), which can be exploited for achieving desirable outcomes like gene modification, gene deletion, or gene insertion depending upon whether an additional template is provided or not during transformation [8, 9]. There are three major categories of these engineered nucleases. The first of these, the Zinc Finger Nucleases (ZFNs) are formed by fusing the DNA binding Zinc finger proteins with an endonuclease called FokI [10, 11]. The second class of SSNs is the Transcription Activator-like Effector Nucleases (TALENs) that have been customized by fusing effector molecules, another class of proteins with DNA binding and recognizing activity, with the FokI endonuclease [12-14]. The most recent class of SSNs is CRISPR/Cas (Clustered Regularly Interspaced Short Palindromic Repeats/CRISPR associated protein) system. In the first two classes of SSNs, proteins are the DNA interacting molecules, whereas, in CRISPR/Cas, simple Watson-Crick Pairing makes an artificially engineered single guide RNA (sgRNA) to bind to its target site, and then Cas produces cleavage to induce a DSB [15]. CRISPR technique also facilitates multiplexing by targeting more than one locus with specific sgRNAs [9]. CRISPR/Cas is an efficient and robust engineering tool, which can help in the improvement of existing crop cultivars and germplasm. Therefore, the present review focuses on the evolution of genome editing techniques and their advantages and disadvantages over conventional techniques for raising climate-resilient crops. We briefly describe various techniques currently being employed for crop improvement and discuss their pros and cons. We further describe how gene editing, with an emphasis on CRISPR/Cas, could be employed for crop improvement and what are its advantages and limitations over other techniques. This review is intended to provide up-to-date information regarding the available methods with respect to their application in the area of abiotic stresses. Additionally, this article is an attempt to critically evaluate the suitability of each method for raising abiotic stress-tolerant plants with respect to time, labour, technical constraints and societal acceptance.

2. CURRENT APPROACHES FOR IMPROVING TOLERANCE TO ENVIRONMENTAL STRESSES IN CROPS

The majority of the crop genotypes that are currently being cultivated have been generated through selective breeding. Most of these crops have been bred for yield-associated traits, although a trend for breeding for stress tolerance has recently emerged. Breeding has contributed massively towards an increase in the quality and yield of crops. This is evident from the impacts of the Green Revolution, which has served to save millions from poverty and hunger [16]. In addition, it is estimated that the Green Revolution saved 17.9-26.7 million hectares of land from being brought into agricultural production [17]. However, the increase in yield for the top four major food crops is currently well below the mark required to meet our estimated demands by 2050 [18]. The use of the more recent nutritionally fortified and pest and herbicide-resistant genetically engineered crops has contributed towards an increase in crop quality and production, but these crops also come with their fair share of limitations, which have contributed to a lack of social acceptance and consequent non-adoption of the technology by most countries of the world. In the following section, we provide a brief description of these techniques which have been employed for raising abiotic stress-tolerant crops. We discuss the methodology, their contribution to agricultural production, and their limitations.

2.1. Plant Breeding

Plant breeding is one of the most important crop improvement techniques being employed. The concept of plant breeding is as old as agriculture and is estimated to date back over 10,000 years to the origin of the domestication of crops [19]. It is the science of deliberately introducing desirable and inheritable changes in plants. Plant breeding includes the selection of plants with better traits and using these superior plants for continued propagation or crossbreeding the superior plants to acquire genotypes with traits of both parents. Modern approaches to plant breeding employ the use of advanced genetics, genomics and molecular biology to obtain carefully selected genotypes with the desired phenotype. However, various factors must be considered prior to any breeding program, such as mode of reproduction, inheritability of genes, mode of action of genes, the magnitude of gene effects, the relationship of gene(s) to agronomic traits, combining ability and screening techniques available [20]. We briefly describe the different breeding approaches being used for crop improvement.

2.1.1. Classical Plant Breeding

Classical plant breeding involves a simple method of 'selection' where plants with superior characteristics are identified from a heterogeneous population and are used for propa-

gation for several generations until those traits get fixed in the population. In simple terms, it is the science of selectively propagating plants with desirable characteristics and eliminating those with undesirable characteristics [21].

Abiotic stress tolerance traits can be found in wild relatives or landraces [20]. The first objective of the breeder is to look for these traits and then utilize them for further generations. One major challenge for this kind of approach is to identify the phenotypes that correlate well with a particular kind of stress. Thus, breeding for any such traits depends upon the screening of maximum genetic variation that is available. Another method is 'Crossing,' *i.e.,* the deliberate interbreeding of closely or distantly related species in order to produce crop varieties with desirable characteristics. The crossing can be intraspecific (within species), interspecific (between two different species), or intragenic (between members belonging to two different genera). However, it is not always necessary that genes from one species integrate into the genome of another so easily. In most cases, resultant offspring are sterile, or offspring do not develop at all. Plants are cross-bred to incorporate desirable feature(s)/trait(s) of one into the genetic background of the other. In rare cases, it can also result in an offspring that performs better than both the parents, a phenomenon called heterosis or hybrid vigour [22]. Classical plant breeding methods are dependent on homologous recombination between chromosomes to generate useful genetic diversity.

There are limitations associated with these breeding methods. First, breeding can be done only in between two plants that are sexually compatible with each other. This limits the traits to be transferred to those only found in existing parental lines. Second, selection can be made only based on genes that give a clear observable phenotype. Another major disadvantage, especially with crossing, is 'linkage drag,' a very common phenomenon where genes which generate unwanted phenotypes are linked to the gene of interest and hence co-inherited. The removal of these unwanted genes usually requires several generations of backcrossing and is sometimes unsuccessful if the genes are closely linked. These factors make classical breeding very laborious, time-consuming, and costly.

2.1.2. Mutation Breeding

Mutation breeding or variation breeding is the process of generating mutagenic plants by exposing their seeds to certain mutagenic agents like Ethyl Methane Sulphonate (EMS) or ionizing radiations (like γ-rays) [23]. These mutagens create random mutations in the genome that can sometimes be beneficial. Mutant populations are then screened, and mutant plants with desirable traits are selected. Mutants can either be selfed or backcrossed to generate desired mutant lines. This method is generally used when desired genes are not found or are depleted in the gene pool and to create novel genotypes. Many genotypes with tolerance to abiotic stresses have been generated using this method. For example, Diamant, a mutant variety of barley, was created by X-ray irradiation of its dormant seeds, which is high yielding, has a good quality of grains, short stem, malting quality, and is resistant to lodging [20, 24]. Similarly, Calrose76, a short stature mutant variety of japonica rice cultivar created by gamma irradiation, was released in 1976 and had more resistance to lodging [25]. More recently, a salt-tolerant rice, Kaijin, was obtained through mutation breeding using EMS as the mutagen [26].

2.1.3. Marker-Assisted Selection or Marker Aided Selection (MAS)

MAS is a process where a trait of interest is selected based on a marker which can be a morphological marker (for example, presence or absence of awn in case of rice), a biochemical marker (for example, allozymes which are different forms of an enzyme coded by different alleles of the same locus), DNA markers [Single Nucleotide Polymorphisms (SNPs), Simple Sequence Repeats (SSRs), Sequence Characterized Amplified Regions (SCAR), Restriction Fragment Length Polymorphism (RFLP), Amplified Fragment Length Polymorphism (AFLP), Random Amplified Polymorphic DNA (RAPD), *etc.*] [27]. DNA markers are PCR (Polymerase Chain Reaction) or non-PCR based. Various molecular markers and their application for QTL mapping in agronomic crops were reviewed by Younis *et al*., 2020 [28]. Screening of plants for clearly visible morphological traits (clearly observable phenotype) like higher yield, bigger fruits, and seeds can be done easily in comparison to more complex traits like biotic and abiotic stress tolerance where each plant has to be tested individually for the presence or absence of the trait among a large number of progenies. MAS selects for markers, especially DNA markers, which are tightly linked to a trait or gene(s) of interest, thereby eliminating the need for phenotypic screening of plants without the marker of interest. In conventional methods, breeding for precise traits is expensive, laborious, and time-consuming. Generally, the greater the complexity of the traits, the more time and efforts are needed to achieve desirable outcomes. Also, the progenies often need to reach maturity before the success of the cross can be determined. MAS overcomes these problems. DNA-based MAS is an effective method for saving time in breeding as it is growth-stage independent, unaffected by environmental conditions, effective to use in early generations, and is efficient when field evaluation is very slow or expensive. There are reports which show the successful use of MAS for generating abiotic stress-tolerant crops. One of these includes the incorporation of 'Saltol,' a QTL for salt tolerance in BR-11 and BR-28, the two mega rice varieties of Bangladesh using marker-assisted backcrossing (MABC) approach [29]. Another example is Swarna-Sub1, which is a submergence tolerant rice variety developed by IRRI in only three years using the MABC approach [30]. In fact, introgression of Sub1, a major QTL for submergence tolerance, is the first successful example of the application of Marker-assisted backcrossing approach in rice for abiotic stress tolerance. Thus, another advantage of MAS is that it allows for the educated selection of correct parental lines for crossing, especially when selecting for introgression of multiple genes, which encode similar traits.

Despite a varied success rate and the general drawbacks of compatibility and linkage drag, breeding for abiotic stress tolerance traits has particularly been difficult because of some major constraints that are associated with abiotic stress. First, the abiotic stress response in plants consists of multiple signal transduction pathways, with a plethora of genes, most of which are not closely linked with each other and are rarely co-inherited after homologous recombination [31]. Except for QTLs, the introgression of multiple genes involved in response to a particular abiotic stress becomes extremely difficult and unlikely. Second, individual plants react differently to similar stress conditions, and it sometimes becomes very tedious to identify promising individual plants and breed a species for more than one resilient trait at a time. Additionally, epigenetic changes play an important role in stress responsiveness by altering chromatin structure at transcriptional and post-transcriptional levels, therefore, understanding epigenetic codes is of great importance for breeding stress-tolerant crops [32]. Often, the performance of genotypes varies each year. Third, various abiotic stresses hardly occur in isolation and are often associated with one another [33]. This is exacerbated by the existence of a very limited number of genetic markers that facilitate tolerance to multiple stresses in various crop plants. These complex traits make a genetic modification of plants for efficient stress tolerance very troublesome to achieve [34].

2.2. Genetic Engineering

Most of the drawbacks associated with breeding approaches can be overcome through the use of genetic engineering methods. Genetic engineering or transgenic technology facilitates the transfer of the desired gene(s) into crops regardless of their source, which makes this method precise, unrestricted by interspecies incompatibility, free from linkage drag and, most of all, less time consuming as compared to breeding methods. Genetic engineering also facilitates the pyramiding of genes for any desired trait(s), thus enabling the generation of crops with multiple desired traits. In fact, genetically engineered crops are the fastest adopted crops for cultivation in recent history, comprising a hectarage of about 191.7 million in 2018, 24 years after their introduction [35]. A large percentage of this is contributed by the herbicide-tolerant and BT crops. However, recently developed genetically engineered drought-tolerant varieties of maize and sugarcane also contribute to the total hectarage of transgenic crops.

Engineering for abiotic stress tolerance requires extensive information on genes that are involved in the stress response. Through functional genomics and genetics, a high number of candidate genes have been identified that are involved in abiotic stress responses, and stress-responsive cellular signal transduction pathways have also been mapped. These studies usually employ the manipulation of the expression of genes. The majority of the studies have employed two main approaches for gene expression manipulation; first, by the overexpression of native/alien genes in the background and second, by silencing the native genes [36]. Some of the genes identified to regulate abiotic stress response in plants using functional genomics include those encoding for ion transporters, antioxidant enzymes, osmoregulatory metabolites, Transcription Factors (TFs), *etc*. These studies have further shown that the modulation of the expression of these genes involved in the stress response has resulted in improved tolerance to abiotic stresses.

2.2.1. Gene(s) Overexpression

Gene overexpression implies the abundant production of a protein of interest in a host by using expression constructs that stimulate the gene's increased transcription. Sometimes, epigenetic modifications can also lead to the abundant production of an endogenous gene transcript [32]. The design is fairly simple. Genes are cloned into plant expression vectors having constitutive, tissue-specific or stress-responsive promoters depending on the need. This cloned gene is then randomly integrated into the genome using different plant transformation methods that are available. The transgenic plants overexpressing the gene of interest are then compared with non-transformed plants for various traits associated with the gene of interest. Various studies have shown that the overexpression of genes, including miRNAs, various Transcription Factors (TFs), major and minor QTLs, which are induced under abiotic stresses, have resulted in increased tolerance in transgenic plants as compared to their wild type counterparts [37, 38]. To mention a few, overexpression of miR169 in tomatoes has resulted in improved drought stress tolerance due to reduced stomatal opening, thereby, resulting in low transpiration rate [39]. Similarly, overexpression of TFs, OsNAC5 and OsNAC14 has resulted in drought stress tolerance and grain yield in rice [40, 41]. Overexpression of glyoxalase enzymes has resulted in enhanced tolerance to salinity, drought and high temperature in rice [42].

2.2.2. RNA Interference (RNAi)

RNA interference is an endogenous mechanism present in plants, which regulates gene expression using small molecules (20-25bp) of interfering RNAs. Mainly, two types of RNAs are involved in RNAi, siRNAs (small interfering RNAs) and miRNAs (microRNAs). These small RNAs are processed from long dsRNA (endogenous or exogenous in origin) and can direct enzyme complexes to bind to mRNAs, resulting in mRNA degradation or inhibition of translation (Post-transcriptional gene silencing, PTGS), thereby down-regulating the expression of target proteins. The cell uses small interfering RNAs as one of its mechanisms for modifying the expression of several genes involved in growth and development, maintaining the genome integrity, and as an immune response against foreign genetic material like viruses. This use of RNA interference has become a powerful tool to inhibit the expression of gene(s) of interest and in functional analysis of genes by creating knockdown transgenic lines. Genes are knocked down using two main approaches, use of exogenously made siRNAs that are transferred directly into the cells or through genetic engineering approaches, which involve the stable integration of expression constructs, which express either antisense RNA, short hairpin RNAs and long hairpin RNAs, that are specific to-

wards a target RNA. The hairpin RNA is processed by the enzyme dicer into several functional siRNAs, which then utilize the endogenous RNA Interference Silencing Complex (RISC) for targeting specific mRNAs. RNAi technology has successfully been utilized to enhance abiotic stresses in various crops. Downregulation of *OsCKX2* using the RNAi technique has resulted in enhanced salinity stress tolerance in rice [43-45]. Comprehensive coverage of the mechanisms of RNAi and its utilization in crop improvement for abiotic stress tolerance has been provided by various reviews and book chapters [46-49].

Despite several reports of enhanced abiotic stress tolerance achieved through genetic engineering, very few of these transgenic crop plants have been successfully translated into viable products for distribution and cultivation. This is because most of the transgenic crops have been generated for functional characterization of genes and possess antibiotic marker and reporter genes, which are required for efficient selection of transgenic lines and molecular studies. Current regulations do not allow for field trials of transgenic crops containing markers and reporters to be tested in fields. Moreover, the generation of marker-free and reporter-free transgenic crops that adhere to current regulations is significantly more tedious and time-consuming. Moreover, the several levels of regulatory testing under laboratory, greenhouse, and field trials required for transgenic crops have made it extremely expensive to develop new transgenic crops. Additionally, the complex nature of abiotic stress tolerance traits, already mentioned above, makes it extremely difficult to engineer abiotic stress-tolerant crops. The additional issue of biosafety concerns has also added to transgenic crops not being accepted as food in many countries.

2.3. Genome Editing for Improvement of Crop Tolerance to Abiotic Stresses

Efficient repair of lesions in the DNA double helix is crucial for maintaining genome integrity and thereby cell survival. Breaks in DNA can occur due to exposure to radiations, reactive oxygen species, mechanical stress, or activity of nucleases. Genome editing technologies exploit the use of existing cell's natural mechanisms to efficiently repair a Double-Stranded Break (DSB) at any site in the genome. DSB can be repaired by two pathways, NHEJ and HDR. NHEJ is an error-prone pathway in which the cell tries to ligate both the ends together and, in this process, can result in indels in the targeted site, especially when overhangs generated by a DSB are not compatible [50]. In HDR, the cell uses a homologous sequence to repair the DSBs, therefore, it is considered as an error-free mechanism [51-54]. Genome editing is a technique for precisely manipulating the genome through the introduction of DSBs at target regions of the genome using SSNs and exploiting erroneous repair by NHEJ, as well as manipulation of the HDR repair for gene disruption and gene insertion, replacement, or modification [55]. Currently, genome editing is employed using three different types of artificially engineered proteins, *viz.* ZFNs, TALENs and the CRISPR/Cas, as mentioned in the introduction section [12, 55-57]. The methods for transformation of the SSNs into plants are those which are currently employed for genetic engineering. However, gene editing techniques have the added advantage that once the genome has been modified in the transformed plants, the inserted transgene (like Cas) can be removed in subsequent generations through segregation.

Plant breeding and the use of genetically modified crops have been successful in increasing agricultural productivity across the globe. However, conventional methods are slow, and even after having enormous potential, a complex regulatory process for GM plants release makes them difficult to be available for food. Genome editing can have three main outcomes, gene modification, gene knockout, or gene insertion. Excluding those with gene insertions, genome-edited plants should not be necessarily classified as transgenic plants; hence they might not have to undergo rigorous regulatory biosafety assessments for release, at least in terms of ecological impacts [58, 59]. These genomes edited plants will have desired mutations, and unlike those acquired through mutation breeding, the exact mutation is known, therefore, no backcrossing is required to eliminate undesired mutations, which generally occurs in mutation breeding. This enables the generation of improved genotypes at a much faster rate as compared to breeding approaches, which also makes the approach more economical. However, the high costs for generating ZFNs and TALENs should not be ignored. Genome editing can be used for gene knockout, which is difficult through genetic engineering. An additional advantage is that gene expression regulation can also be achieved through editing/mutation of gene regulatory regions such as promoters. Targeted insertion of transgene *via* HR in case of gene editing also minimizes the rare hazards associated with the transgenesis approach, where random insertion of the transgene can disrupt gene functions or the formation of new Open Reading Frames (ORFs) encoding toxic proteins. Stacking different transgenes at a single site is also possible, which can be moved to other germplasm as a single locus [60]. This is particularly important for the generation of multiple abiotic stress-tolerant plants. Various genes regulating different abiotic stresses can be simultaneously targeted, theoretically enabling the generation of plants with a tolerance to multiple abiotic stresses.

2.3.1. Zinc Finger Nucleases (ZFNs)

ZFNs are fusion proteins consisting of a target site-specific DNA binding domain and a DNA cleavage (nuclease) domain attached *via* a 21 aa long linker. The DNA-binding domain of Zinc finger nucleases recognizes the DNA. This belongs to a functionally diverse group of proteins characterized by at least one stabilizing zinc ion coordinated to its structure. The zinc finger domain primarily used in ZFNs consists of approximately 30 amino acids with a conserved ββα fold and binds a single zinc ion. The α-helix residues contact the major groove of DNA. The DNA binding domain of ZFNs comprises a set of Cys2His2 Zinc fingers, usually 3 to 6 in number and each zinc finger motif recognizes 3 base pairs on a DNA strand, therefore, arrays of 3-6 recognise 9 or 18 bp of DNA, respectively. The nuclease domain is derived from a sequence-independent *Flavabacterium*

okeanokoites restriction enzyme FokI, which is composed of two domains, a DNA binding domain that recognizes 5'-GGATG-3' sequence and a cleavage domain that cuts downstream of the recognition sequence on both strands. This enzyme requires dimerization for its activity; therefore, to specifically recognize a site, two FokI monomers are linked to two sets of Zinc Finger Arrays (ZFAs) directed to adjacent sequences on opposite strands with a spacer region of 6-25 bp in between. FokI catalytic activity results in cleavage in the spacer region between two ZFN units, resulting in a staggered DSB with overhangs [61]. Zinc finger domains have been designed to recognize all 64 possible nucleotide triplets. The reagents, protocols, and software for engineering ZFNs are publicly available. The web-based ZiFiT (Zinc Finger Targeter) tool can be used for the identification of potential ZFN target sites in the locus of interest [62]. Modular assembly, Oligomerized Pool Engineering (OPEN) and Context-Dependent Assembly (CoDA) are other available tools. Reviews by Weinthal *et al*. [63] have superbly highlighted the various reports which show the mechanism and use of ZFNs for incorporating different types of point mutations, small and large deletions and insertions in plants. However, despite these reports on the success of ZFNs for gene editing in plants, there have been limitations, which have impeded their use for crop improvement. One of the major limitations is the context dependency of zinc-finger nucleases. Recognition of a nucleotide triplet is dependent on adjacent zinc finger proteins, which make the modular assembly of these nucleases a difficult task to achieve.

2.3.2. TALENs

TALENs is another class of SSNs composed of a DNA binding domain made up of Transcription Activator-Like Effectors (TALEs) and a DNA cleavage domain made up of type IIS bacterial endonuclease FokI enzyme. TALEs are virulence factors secreted by species of plant pathogenic bacteria, *Xanthomonas,* that binds to Effector Binding Elements (EBEs) in host gene promoters to activate gene expression [12]. An effector molecule consists of an N-terminal Translocation Domain, C-terminal Nuclear Localization Signal (NLS), Transcription Activation Domain, and a central DNA Binding Domain. DNA binding domain of effector is composed of a tandem array of 12-30 identical repeats of 34 amino acids long and specific Repeat Variable Diresidues (RVDs) present at amino acid positions 12 and 13. Structural analysis of the effector molecule has revealed that its RVD loop contacts the major groove of DNA. The amino acid at the 13th position recognizes the target nucleotide on the sense strand of DNA, while amino acid at the 12^{th} position stabilizes this structure through hydrogen bonding [64]. For each of the four different nucleotides, there are four specific RVDs. RVDs and their sequences determine the TALE DNA binding specificity. TALENs can, therefore, be designed virtually for any DNA sequence based on this predictable one-to-one relationship (one repeat for one nucleotide), which can be summarized in a simple code. The most widely used RVDs are NI (Asn, Ile), which recognizes Nucleotide A, G by NN (Asn, Asn), C by HD (His, Asp) and T by NG (Asn, Gly) [65]. Like ZFNs, TALENs are designed as a pair separated by a spacer sequence as FokI nuclease is only active on dimerization. One of the major advantages of this technology is the specificity of TALEs due to these RVDs, which mediate one to one interaction, and context-dependence effects on DNA binding and specificity is rare. Various software are available for the design of TALEs and TALENs and these have clearly been described by Khan *et al*. [66]. They have also comprehensively illustrated the successful implementation and potential of TALENs in crop improvement. TALENs are a significantly better and improved version of genome editing tool but present a high cost associated with protein engineering, and their large size make them challenging for delivery into the cells [57].

2.3.3. CRISPR/Cas System

CRISPR was reported in *Escherichia coli* genome, in 1987, when Ishino and his coworkers accidentally cloned a part of the cluster of interrupted direct repeats along with the *iap* gene [67]. In 2002, Jansen and his colleagues named it CRISPR (Clustered Regularly Interspaced Short Palindromic Repeats) and also identified four CRISPR-associated (Cas 1-4) genes. Following that, different CRISPR/Cas subtypes and Cas protein families were discovered [68]. In 2005, three independent groups found that CRISPR spacers are acquired from phage and plasmid DNA, however, its biological function was still unclear at that time [69-71]. In 2007, Barrangou and his coworkers reported that CRISPR is a part of the defence system of bacteria where bacteria accumulate traces of any infection by adding the genomic part (in the form of spacers) of invading viruses and plasmids as a memory. Later, these homologous sequences can be used for destroying foreign DNA elements [72]. Until now, the CRISPR system has been reported in almost 50% of the sequenced bacterial genomes and approximately 90% of the sequenced archaeal genome.

There are two main classes of CRISPR/Cas systems, class I and class II. Class I forms a CASCADE (CRISPR associated complex for antiviral defence) complex with multiple Cas proteins to mediate interference with foreign nucleic acids. Class II employs a single large Cas protein having multiple domains for this purpose. These two classes are further sub-divided into six sub-classes. Type I (Cas3), III (Cas10), IV (Csf1) comes under class I, whereas type II (Cas9), V (Cas12a, Cas12b, Cas12c, Cas12d/CasY, Cas12e/-CasX), VI (Cas13a, Cas13b, Cas13c, Cas13d) comes under class II [73-80]. Further, these types are divided into 33 subtypes based on the architecture of the CRISPR array and the presence of signature genes that can only be found in a particular subtype. An AT-rich leader sequence is present at the CRISPR locus, which is composed of direct short repeats (28-37 bp) interrupted by unique spacers (32-38 bp) [81]. In a CRISPR array, usually less than 50 units of repeat-spacer sequences are present. Small clusters of Cas genes are also located near CRISPR arrays.

CRISPR-mediated defence mechanism in bacteria is divided into three main stages; adaptation, crRNA biogenesis

and target interference. In the adaptation stage, invading nucleic acid (protospacer) is incorporated in the CRISPR array in the form of spacers. Target sequences (spacers) for integration into the CRISPR locus is not a random process. A short sequence present next to a protospacer called Protospacer Adjacent Motif (PAM) is important for spacer acquisition in some CRISPR systems (type I, II, V). This is followed by crRNA biogenesis, where the CRISPR array is then transcribed into a long precursor CRISPR RNA (pre-crRNA) that is further processed to generate mature crRNA, which guides the nuclease protein to degrade invading nucleic acids. This is followed by interference, where mature crRNAs act as a guide to specifically bind with invading nucleic acids.

2.4. CRISPR/Cas9 as a Genome-editing Tool and its Molecular Mechanism for DNA Targeting

In 2012, the use of CRISPR/Cas as a genome-editing tool was established when it was shown that target specificity can be changed by changing the 20 nucleotides of crRNA. The most adaptive and widely used system for genome editing is a type II CRISPR/Cas9 system from bacteria, *Streptococcus pyogenes* [15]. In the native system, Cas9 requires two RNAs, *i.e.,* a crRNA (20 nt) and tracrRNA (trans-activating crRNA, 80bps), where crRNA determines target specificity and tracrRNA stabilizes the whole structure, thereby activating crRNA guided target sequence cleavage by Cas9 [82]. This three-component system has been engineered into a two-component system by fusing both the RNAs into a chimeric single guide RNA (sgRNA) that works well with the Cas9 enzyme [15]. For cleavage, Cas9 requires a short-conserved sequence (PAM), 5'-NGG-3' or less frequently 5'-NAG-3', which must be present downstream of the target sequence.

In principle, ZFNs and TALENs can do whatever is achievable using CRISPR/Cas. Nevertheless, because of its relative simplicity, cost-effectiveness, and versatility, CRISPR/Cas has been used for genome editing in a wide variety of plant and animal species. RNA recognition of DNA rather than protein recognition of DNA in the case of ZFNs and TALENs makes it a more simple technology to use. Protein engineering is a complex, expensive, and time-taking process. In CRISPR/Cas, only 20 nts of sgRNA are needed to target different DNA sites. Another major advantage is that it facilitates multiplexing. Multiple gRNAs with a single Cas9 protein can be used to target a single gene or many different genes as opposed to ZFNs and TALENs, where for each target site, a separate ZFN or TALEN is required. CRISPR constructs are also smaller, therefore, easier to deliver than ZFNs or TALENs. Besides these, due to the intrinsic property of the CRISPR/Cas system, it can recognize methylated DNA, therefore, it can be used to target such sites that ZFNs and TALENs cannot target.

There are two methods for delivering CRISPR/Cas9 constructs in plant cells. One is the transient gene expression method in which pre-assembled Ribonucleoproteins (RNPs) of purified Cas9 protein and *in vitro* transcribed guide RNA is delivered into the cells. Alternatively, mRNA that expresses Cas9 along with the gRNA is also used. Microinjection, electroporation, liposome and Polyethylene Glycol (PEG) mediated transformation, Mesoporous Silica Nanoparticles (MSN) and cell-penetrating peptides methodologies are used for transforming cells [83]. This method of delivering CRISPR constructs is safe as RNPs get degraded by cells reducing the off-target effects of Cas9 and making it a transgene-free technology [84]. Another method is the stable gene expression in which an all-in-one vector carrying gRNA cassette and Cas9 gene or binary vector carrying each one of them on two different plasmids is used (Genome editing by CRISPR/Cas9 and different methods of delivery of constructs is depicted in Fig. **1**). Vectors are delivered by electroporation, microinjection, heat shock methods or *Agrobacterium*-mediated transformation. Due to the stable integration of transgenes, plants developed by this method are considered transgenic by some, though transgenes can be eliminated by genetic segregation to make them transgene free.

2.5. Alternatives to Cas9 for DNA Targeting

Enzymes from bacteria like Cas12a (Class II, type V), formerly Cpf1 (CRISPR from *Prevotella* and *Francisella*) are good alternatives to Cas9. Cpf1 expands the range of target sequences as it recognizes T-rich PAM (5'-TTN-3') instead of G-rich in the case of Cas9. Cpf1 does not need tracrRNA for target site recognition and cleavage, therefore, a single crRNA (42 nts) is capable of editing, making the design and delivery of this genome-editing tool easier. Cpf1 has a single nuclease domain resulting in a staggered cut with 5' overhang. This feature is particularly useful for HR-mediated gene editing as ends become predictable. Another advantage is that Cpf cleaves the target sequence at the distal end from the PAM instead of the proximal end, as in the case of Cas9, creating a possibility for subsequent rounds of cleavage by cpf1 [85]. The mechanism of action of the Cpf1 (Cas12a) enzyme is given in Fig. (**2**). Cas12a from *Acidaminococcus* sp. (AsCpf1), *Lachnospiraceae* bacterium (LbCpf1) and *Francisella novicida* (FnCpf1) have been used successfully for targeting rice, Arabidopsis, tobacco, tomato, citrus and cotton [34, 86-95].

In addition to gene knockout and insertion and relying on endogenous repairing pathways for desirable mutation, partially inactive nickase Cas9 (nCas9) or catalytically dead Cas9 or cpf1 (dCas9/dCpf1) have also been used for precise base editing by fusing them with a base editor cytidine base editor like APOBEC1 [96, 97] or adenine base editor like E. coli TadA [98]. Successful base editing for trait improvement is reported in rice, wheat, maize, and tomato [99-104].

2.6. Gene Regulation by Crispr

Besides DNA targeting, the CRISPR technique has been expanded to diverse applications (Fig. **3**) in the last few years. The dCas9/dCas12a are used as "gene switches" for the control of gene expression. First-generation dCas9 tool for gene regulation involves the use of fusion protein of

dCas9 with an activator (CRISPR activation or CRISPRa) or repressor (CRISPR interference or CRISPRi). With reportedly low efficiency of activating and silencing genes by first-generation dCas tools, scientists have come up with second-generation tools (SunTag, SAM, VPR, ScRNA) having better gene activation potential and multiplexed regulation. In addition to first- and second-generation tool, a ligand inducible gene regulation toolbox (Dimerization systems, split dCas systems, receptor-coupled systems) enabling temporal and spatial control of gene expression have been developed, which comes under the category of third-generation dCas tool [105, 106]. Besides dCas9/dCas12a, Cas9 protein can also be used for gene regulation by targeting regulatory elements like promoters, transcription factors, and enhancers using NHEJ or HDR method.

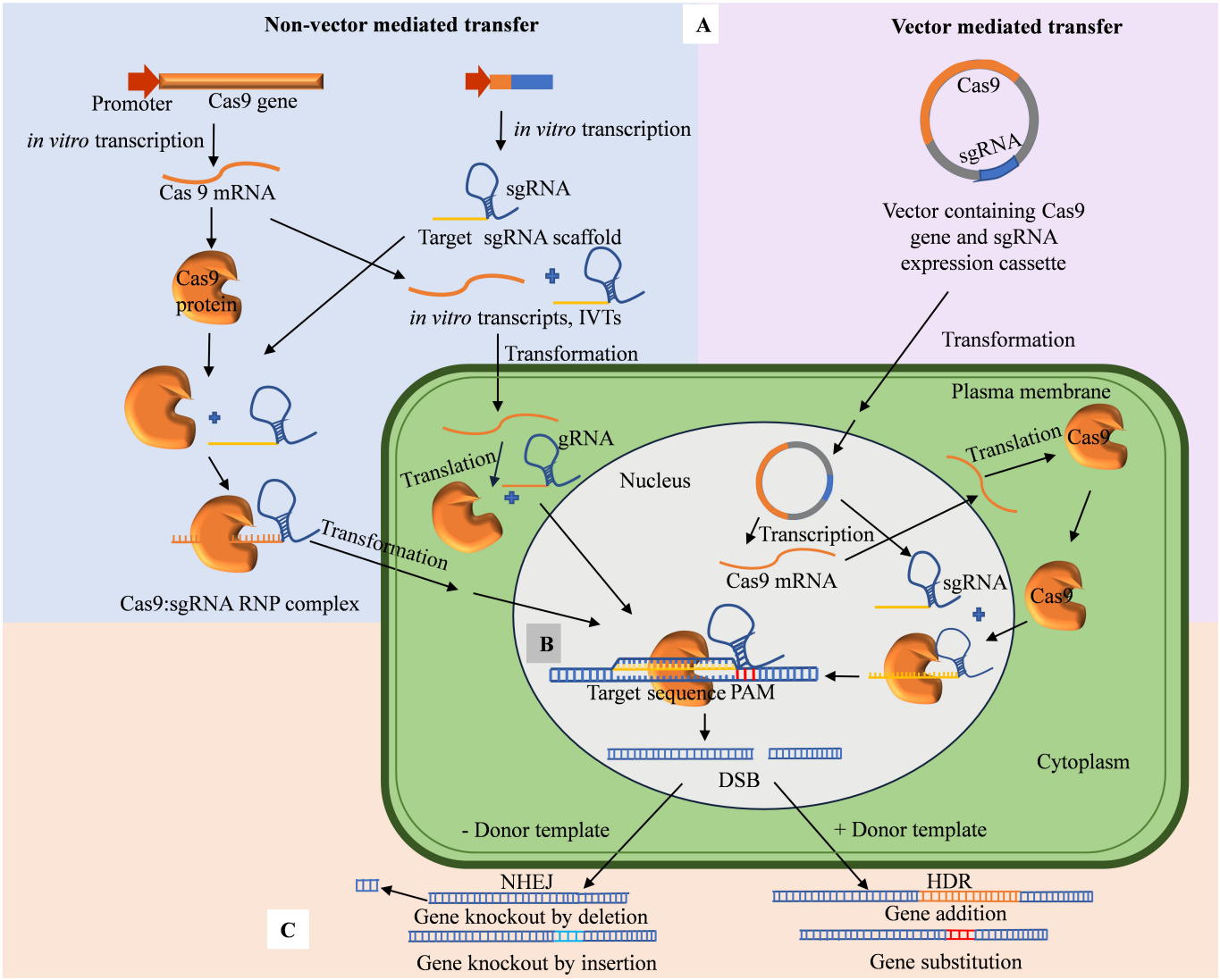

Fig. (1). Overview of genome editing and various methods of CRISPR/Cas9 delivery in plant cells. CRISPR/Cas9 constructs are delivered mainly by two methods; Non-vector mediated and vector-mediated. (**A**) In non-vector mediated delivery, pre-assembled Cas9 and sgRNA Ribonucleoproteins (RNPs) or *in vitro* transcribed Cas9 mRNA and sgRNA (IVTs) are used. These are expressed for a short duration in the cell, therefore, the off-target effects are minimum. In vector-mediated delivery, an all-in-one plasmid containing sgRNA and Cas9 gene or binary plasmid is used. Cells are then transformed by the Agrobacterium-mediated method or particle bombardment. (**B**) Inside the nucleus, after PAM (Protospacer Adjacent Motif) recognition and binding to the target DNA site, Cas9 produces a Double-Stranded Break (DSB), which gets repaired by endogenous repairing pathways and depending on whether an additional donor template was provided or not at the time of transformation, it can have several outcomes. (**C**) In the absence of any donor DNA, Non-Homologous End Joining (NHEJ) can disrupt the target gene function by random insertion and deletions of few base pairs causing frameshift mutations. With donor DNA having homology to the target site, the Homology Directed Repair (HDR) mechanism of endogenous repair can be exploited to achieve gene substitution or gene addition at the target site. (*A higher resolution / colour version of this figure is available in the electronic copy of the article*).

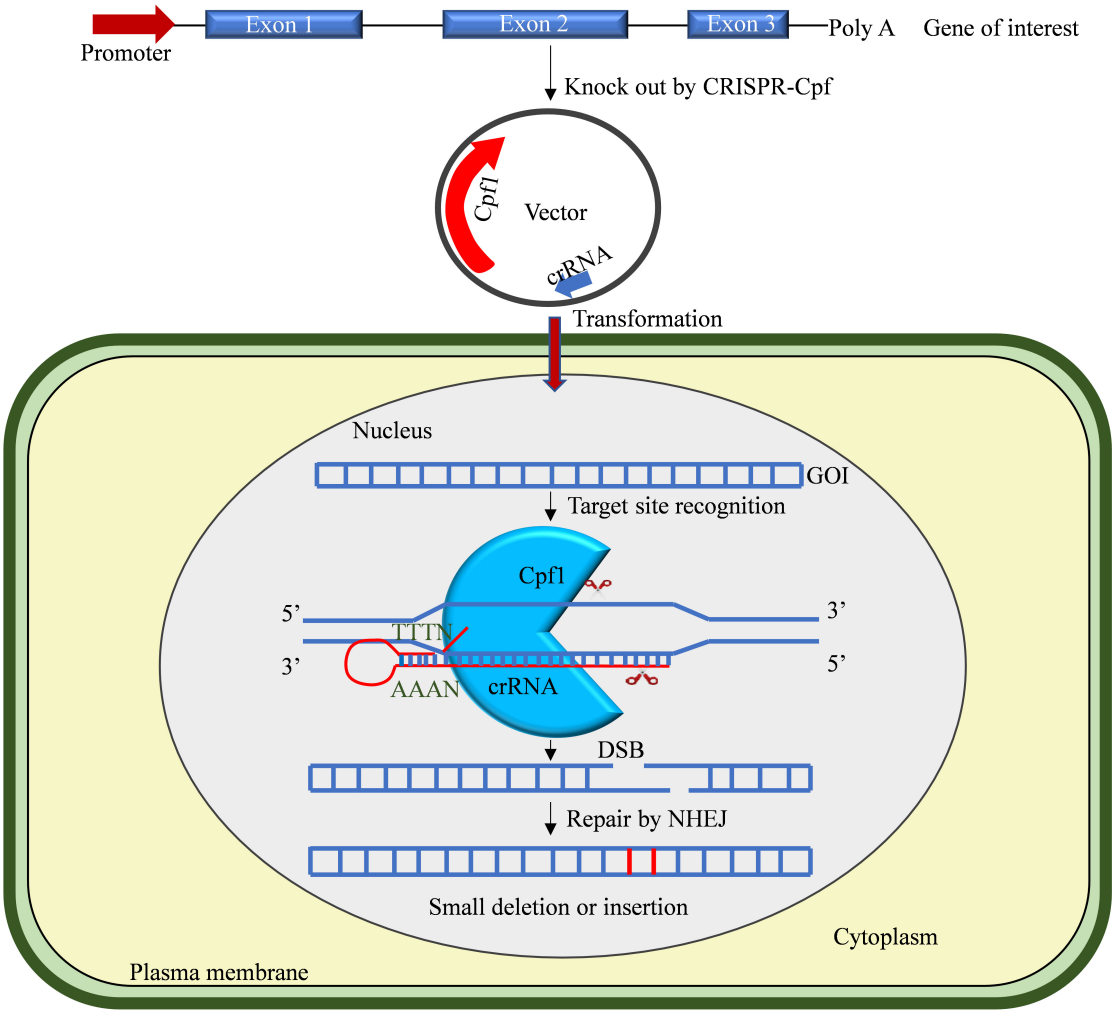

Fig. (2). CRISPR/Cpf1 mediated targeting of Gene of interest (GOI); CRISPR from *Prevotella* and *Francisella* 1 or CRISPR/Cpf1 (now known as Cas12a) is an enzyme similar to Cas9 that belongs to the Class II RNA-guided endonuclease system. Cpf1 expands the range of target sequences as it recognizes T-rich PAM (5'-TTTN-3') instead of G-rich in the case of Cas9, and it does not need tracrRNA for target site recognition and cleavage, therefore, a single crRNA (42 nts) is capable of editing, making the design and delivery of this genome editing tool easy. Cpf1 has a single nuclease domain resulting in a staggered cut with 5' overhang, and it cleaves at the distal end from the PAM instead of the proximal end as in the case of Cas9, creating a possibility for subsequent rounds of cleavage by Cpf1. The detailed mechanism of action by Cpf1 is shown in the diagram. (*A higher resolution / colour version of this figure is available in the electronic copy of the article*).

CRISPR from type III (both DNA and RNA) and type VI (only RNA) systems recognises RNA, thereby enabling the RNA-guided RNA targeting, detection, capture and tracking. The enzyme from *Leptotrichia shahii*, LshC2c2 (Class II, type VI) with a 22-28 nt long crRNA and a protospacer flanking sequence (PFS) recognizes and cleaves RNA; this RNA guided RNase property of C2c2 (now known as Cas13a) is due to the presence of two higher eukaryotes and prokaryotes nucleotide-binding (HEPN) domains. All functionally characterized proteins with HEPN domains are RNases. This RNA-guided RNase property of class II, type VI system from *Leptotrichia shahii* has paved the way for the development of programmable nucleases that can be used for *in vivo* RNA manipulation [107]. This RNA targeting property of C2c2 can be exploited for cleavage, correcting gene transcripts, organellar RNA editing, generating transcript variants. Cas9 or Cas13 based RNA targeting systems are generally very large (>1100 amino acids) [108] and even larger when fusion proteins are used, which makes their delivery in the plant system very difficult. Recently, a smaller version of Cas13, *i.e.,* Cas13d (930 aa), has been discovered [108-111], which is by far the smallest of all available RNA targeting CRISPR enzymes (20% smaller than Cas13a-c) [112]. In plants, the activity of LshCas13a has been demonstrated in *Nicotiana benthamiana* and Arabidopsis [113, 114] and orthologue of Cas13 from *Leptotrichia wadei* Lwa-Cas13a has been used in rice protoplast [107].

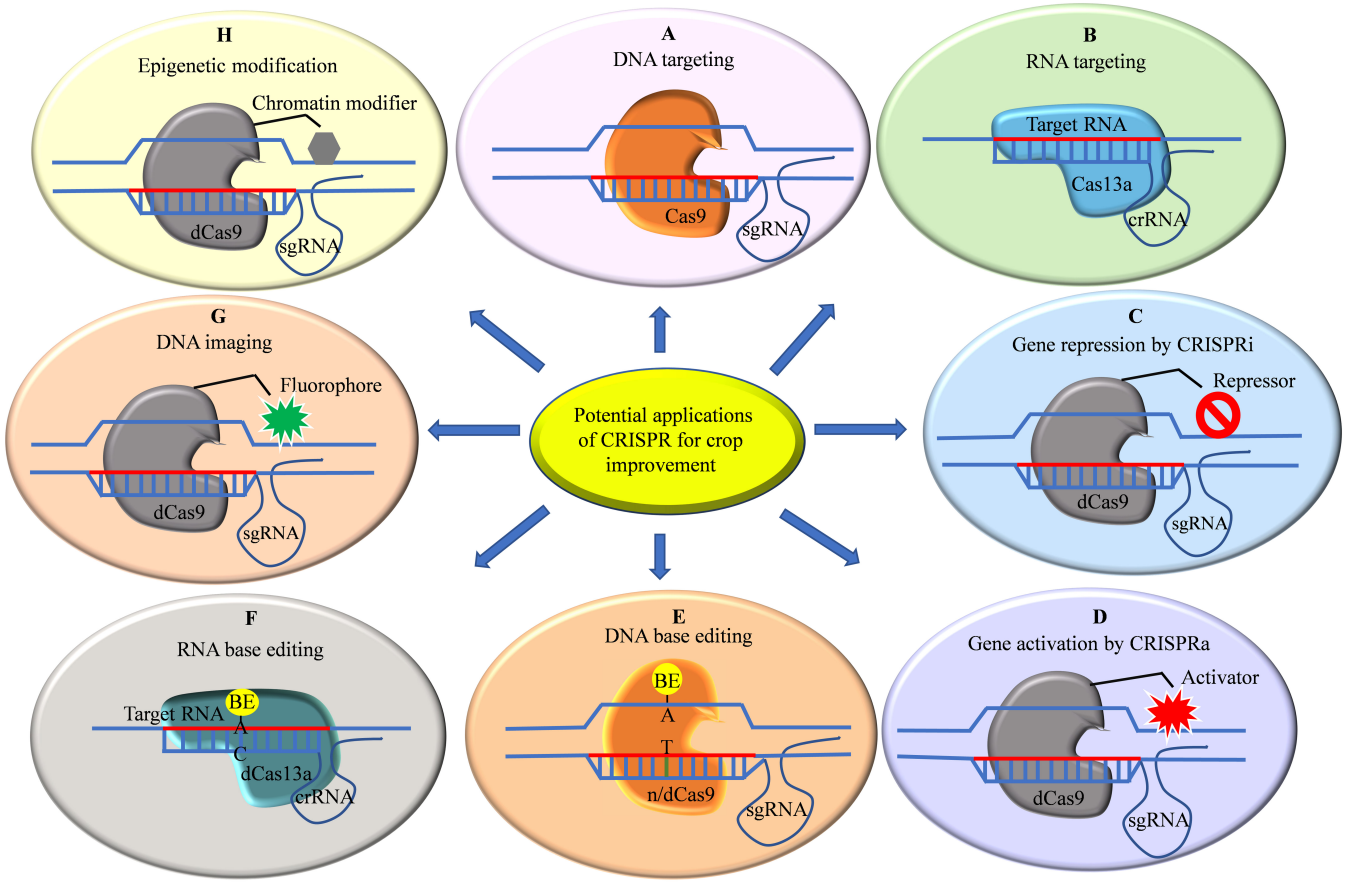

Fig. (3). Potential applications of CRISPR for crop improvement; (**A**) CRISPR is used for DNA targeting. This can be of particular importance for targeting a specific member of a multigene family. Also, genes associated with different traits can be targeted simultaneously by multiplexing specific sgRNAs. Besides this, (**B**) it was recently discovered that Cas13a has the potential of targeting and cleaving RNA; (**C**) CRISPR interference (CRISPRi) and (**D**) CRISPR activation (CRISPRa) techniques can be used for regulating gene expression by using a repressor or activator protein fused with dead Cas9 (dCas9). (**E**) Fusion of dead Cas9 or nCas9 with a base editor can lead to a base change in DNA. A base editor like adenine deaminase will deaminate adenine to convert it into inosine, which will be recognised as a G during DNA synthesis resulting in G:C in place of A:T. (**F**) Similarly, Cas13a can be used for RNA base editing; (**G**) Fusion of Cas9 with a fluorophore can be used for live DNA imaging to study chromatin organisation during the cell cycle. (**H**) Epigenetic modifications at desired genomic loci can be achieved using Cas9-chromatin modifier fusion protein. (*A higher resolution / colour version of this figure is available in the electronic copy of the article*).

3. USE OF CRISPR FOR PLANT GENOME EDITING: CHALLENGES AND SOLUTIONS

CRISPR has been engineered for diverse applications and provides a great platform for plant genome modification. A diagrammatic representation of different methods of crop improvement is given in Fig. (**4**). However, there are several limitations associated with this technique; some are common to all organisms while some are specific to plant systems. Constant efforts are being made by scientists all over the world to overcome these limitations.

(a) One of the major limitations of this system is the high frequency of off-targeting effects [115]. For target site recognition and cleavage by Cas9, a 12 nucleotide seed region of sgRNA is very important. However, this system allows some relaxation in the distal site from the PAM site (non-seed region), where mismatches can be tolerated [116, 117]. This is one mechanism by which a bacterial system ensures that invading DNA with point mutations does not escape the immune system [118]. This limitation has been overcome by many strategies. Choice of sgRNA is very important, therefore, many publicly available online tools are available, which can help in selecting unique target sites for sgRNA designing. Moreover, by deactivating one of the nuclease domains of WT Cas9, scientists have converted it into nickase (nCas9). Paired nickases are used in combination with two different gRNAs to target a single site resulting in a staggered DSB at the target site, also increasing the chance of target gene disruption [119]. Another method is the use of fusion protein of FokI and dCas9 (dCas9-FokI). Dead Cas9 (dCas9) is made by deactivating both the nuclease domains, which can only recognize the target sequence but cannot cleave DNA. This dual enzyme fusion protein with two

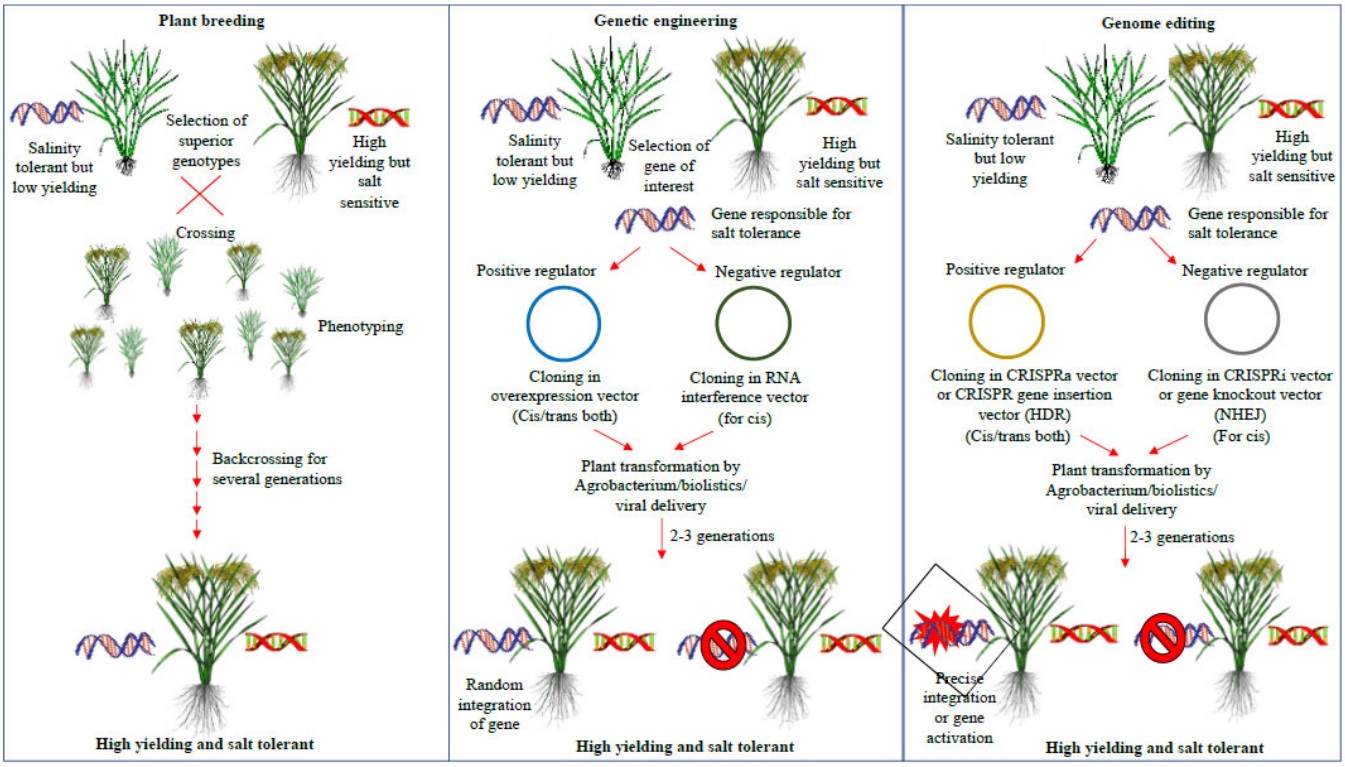

Fig. (4). Schematic diagram showing the evolution of breeding technology from conventional to modern-day genome editing used for crop improvement. (**A**) Plant breeding primarily requires the selection of parental genotypes followed by recurrent backcrossing to obtain the desired progeny with a trait of interest. (**B**) Genetic engineering is based on the identification and isolation of the gene of interest controlling the desired trait. This is followed by the genetic transformation of the crop to be improved. (**C**) Genome editing using CRISPR is gaining importance due to its unique feature of precision in modifying/replacing/silencing/addition of the gene of interest. (*A higher resolution / colour version of this figure is available in the electronic copy of the article*).

gRNAs can bind to two precisely disposed half-sites like ZFNs and TALENs, resulting in less off-targeting [120]. Positively charged amino acids are present in a groove between RuvC, HNH and PAM interacting domains of Cas9, which can bind to the non-target strand of target DNA by substituting a positively charged amino acid with alanine; Cas9 has been converted into "Enhanced SpCas9" (eSpCas9) with reduced off-target effects [121]. Off targeting can also be reduced by regulating the expression of Cas9 temporally and spatially, for example, by using tissue-specific promoters or photo-activable Cas9 (paCas9). The paCas9 consists of split Cas9 fragments that get dimerized on exposure to light; this optogenetic control offers a nice way for the spatiotemporal regulation of RNA-mediated genome editing [122]. SpCas9 variants like VQR, VRER, EQR, QQR1, D1135E [80, 123, 124] that recognizes different or longer PAM sequence have been designed, resulting in more specificity for its target sequence. For increasing the accessible target sites and specificity, Orthologues of Cas9 from different bacteria like *Staphyloccous aureus* Cas9 (SaCas9), *Streptococcus thermophilus* Cas9 (St1Cas9 and St3Cas9), *Neisseria meningitis* Cas9 (NmCas9), *Campylobacter jejuni* Cas9 (CjCas9), *Brevibacillus laterosporus* Cas9 (BlatCas9) have been used for mammalian genome editing and only some for plant genome editing [125-129]

(b) Second limitation is the temperature sensitivity of Cas9 that is the most commonly used genome-editing tool. Its editing efficiency increases at a higher temperature. Likewise, Cas12a also works better at higher temperatures [80].

(c) Another problem is the difficulty of removing transgenes from stably integrated constructs. Although it can be removed by breeding and screening of segregated populations, the situation becomes more complicated in the case of vegetatively propagated, self-incompatible and polyploid plants. Therefore, RNPs or plasmid-mediated transient expression of the CRISPR construct is best in these cases. Despite its low efficiency of editing, CRISPR-Cas RNPs have been used successfully for targeting the genome of *Arabidopsis*, rice, wheat, lettuce, wild tobacco, apple, *Petunia*, grape and soybean [130-135]. Very recently, a transgene killer system has been used in rice by linking suicide transgenes along with the CRISPR constructs. All transgenes will get eliminated in the T1 generation [136].

(d) sRegeneration of plants from protoplasts is still a big challenge for plant scientists. Also, lengthy tissue culture procedures generate undesirable somaclonal variations [80]. Another problem is achieving multiallelic editing in the case of polyploid species [137]. Despite these limitations, the ad-

vantages of CRISPR over conventional strategies cannot be neglected, and ongoing rapid research and improvements in the technique will help in generating crops with improved traits in a shorter period of time. Successful examples of raising abiotic stress-tolerant crops are given in Table **1**.

CONCLUDING REMARKS AND PROSPECTS

Humans have been engaged in developing improved cultivars of various crop species since the beginning of civilization. Conventional methods of crop improvement through plant breeding are a cumbersome and time-taking process. With the current technologies, it has become possible to sequence genomes of various economically important crops. Moreover, much of the information about loci (QTLs) responsible for a particular trait and their relative contribution has been obtained. Availability and ease of retrieving data about the genome of various crops have provided a platform for achieving goals of improved cultivars in a short span of time. Although genetic engineering has speeded up the process of crop improvement, strict regulations for the release of GM crops restrict their global application. The use of genome editing tools has marked the beginning of a new era. Various genome-editing tools (ZFNs, TALENs, CRISPR) are now available. However, they have not been utilized to their full potential, especially in the case of plants. Genome editing tools have the potential for overcoming most of the limitations of plant breeding and genetic engineering approaches. Advanced breeding methods have helped scientists to quickly manipulate the crops with the trait of interest. Among various methods available, CRISPR/Cas has the enormous potential to bring a new green revolution for developing climate-smart crops. All it needs is genome sequence information of the host, and then any site in the genome can be targeted easily. Their role is limited to the generation of a DSB at the target site; then the endogenous repairing pathways of the cell are responsible for target site modification. In this way, it resembles the mutations that occur naturally in the cell. Though, nature of mutations is not in control of a researcher, screening for selecting the useful ones can be easily done. A comparison between plant breeding, genetic engineering and genome editing is given in Table **2**. Future areas of research include the development of robust transformation methods, designing of easy-to-deliver and efficient vectors, improvements in tissue culture methods to increase the applicability of these tools for different crop species. Moreover, a better understanding of the DNA repair machinery of cells is required to increase the mutagenesis efficiency of these tools.

Table 1. Representative examples of CRISPR/Cas9 mediated improvement in crops for abiotic stress tolerance

Plant Species	Target Gene and Function	Target Trait	Type of Modification	Repair Mechanism	Transformation Method	Achievement/ Outcome	Refs.
Arabidopsis thaliana	UGT79B2 and UGT79B3 involved in sugar transportation	Cold, salt and drought tolerance	Knockout	NHEJ	*Agrobacterium* mediated floral dip	Abiotic stress tolerance by the modulation of anthocyanin accumulation	[138]
Oryza sativa	OsRAV2, involved in the development	Salt stress tolerance	Knockout	NHEJ	*Agrobacterium* mediated	Reported the involvement of OsRAV2 in salt stress response	[139]
Oryza sativa	OsDST, encodes a zinc finger TF	Salt and drought tolerance	Knockout	NHEJ	*Agrobacterium* mediated	Abiotic stress tolerance by broad leaf area and reduced stomatal density	[140]
Solanum tuberosum	StMYB44, involved in phosphate uptake by roots and its distribution	Phosphate stress response	Knock out	NHEJ	Agrobacterium mediated	Reported StMYB44 as a negative regulator of Pi transport	[141]
Zea mays	ARGOS8, a regulator of ethylene response	Drought tolerance	Insertion/replacement	HDR	Particle bombardment	Improved growth and productivity under drought stress	[142]

Table 2. Comparison of various methods of crop improvement.

Parameters	Plant Breeding	Genetic Engineering	Genome Editing
Methods employed	Crossing of hundreds or thousands of genes to get the desirable combination in the progeny	Precise transfer of gene(s) (either native or alien) in an organism's background	Modification of existing genes or gene substitution or addition to get desirable outcomes
Working principle	Crossing and selecting individuals with a superior gene pool	Gene(s) are cloned in an expression vector and are transformed in the host *via* different transformation methods available	Using protein (ZFNs, TALENs) or RNA (CRISPR/Cas9) recognition of DNA and a non-specific endonuclease DSBs are introduced in a target site, which can be modified by exploiting endogenous repair pathways
Barrier across species	The gene pool can be derived from the same species or closely related species	Gene(s) from any source (plant, animal, microbe) can be introduced	Endogenous gene(s) can be modified or substituted or gene(s) can be added irrespective of their source

(Table 2) contd....

Parameters	Plant Breeding	Genetic Engineering	Genome Editing
Accuracy of gene transfer	Little or no guarantee of achieving the desirable combination of genes even in hundreds and thousands of crosses. Often, undesirable genes along with the desirable ones get transferred, sometimes resulting in linkage drag	Only selected gene(s) are transferred	Selected gene(s) are modified or transferred
Integration in genome	Genes are transferred from parents to progeny in an ordered way; the natural mechanism of transfer of genes from parents to progeny	Genes are randomly integrated, which can activate or repress other genes	Precise modification or integration of gene(s)
Multiplexing	A complex process as it needs extensive crossing and selection	Easy to stack different genes in a single plant	Multiple genes can be targeted simultaneously
Time	Time-consuming;it takes several generations	Desirable changes can be achieved in few generations (three)	Desirable changes can be achieved in few generations (three)
Expenditure involved	Uses relatively inexpensive methods for phenotypic assessment	Current regulations and several levels of testing and field trials for the release of transgenic crops makes it an expensive technology	Less costly alternative to GMOs. Not all products developed by this method can fit into the true definition of GMOs.
Social acceptability	It is a traditional method of crop improvement	In some countries, these are acceptable.	Rules and regulations are yet to be decided.
Final product	The final product is an improved genotype developed by the recombination of genes from parental lines.	The final product is genetically engineered, usually consisting of a gene expression construct integrated into the genome.	Except for gene insertions, the final product can be transgene free.

CONSENT FOR PUBLICATION

Not applicable.

FUNDING

The study received research grants from ICGEB, New Delhi, India and SERB-Power Fellowship Award (SPF/2021/000013).

CONFLICT OF INTEREST

The authors declare no conflict of interest, financial or otherwise.

ACKNOWLEDGEMENTS

SLS-P acknowledges research grants received from ICGEB, New Delhi, India and SERB-Power Fellowship Award (SPF/2021/000013). YG acknowledges the Senior research fellowship received from CSIR.

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