Concepts and Strategies in Plant Sciences
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Anjanabha Bhattacharya
Vilas Parkhi
Bharat Char *Editors*

CRISPR/Cas Genome Editing

Strategies And Potential For Crop Improvement

Springer

Concepts and Strategies in Plant Sciences

Series Editor

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Editors

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Preface

CRISPR/Cas (**C**lustered **R**egularly **I**nterspersed **S**hort **P**alindromic **R**epeats and **Cas** for CRISPR-associated proteins) is a widely used genome editing (GE) technique among a group which are often collectively referred to as new plant breeding technologies (NPBTs) and can demonstrably have an impact on crop production. CRISPR/Cas can be used as a tool for creating improved crop plants for enhancing the agricultural output and availability of nutrition. Increasing farmer incomes and agricultural productivity to meet the needs of the world's population, which by some estimates, may touch 9.7 billion (bn) by 2020, is a herculean task. Further, climate change, reduced arable land under crop cultivation, water scarcity and reduced labour availability in the agricultural sector will make food production more challenging than ever. GE involves precise alteration of gene sequences without proven unwarranted effects in crops. This book mainly focuses on CRISPR/Cas technology. Among many GE technologies available, CRISPR/Cas, is becoming a tool of choice, due to its simplicity, versatility, scalability and lower cost when compared to other biotechnological tools. By some market estimates, plant breeding and CRISPR/Cas plant market may reach to US $14.55 bn by 2023 thus emphasising the vast potential of this technology in years to come. Random mutagenesis based genome editing through physical irradiation and chemically induced, has been accepted all over the globe but is time-consuming and therefore, may not fulfil emerging market demand. It is, therefore, expected that CRISPR/Cas genome editing will be perceived more positively and therefore, this book is timely. Though traditional breeding has played an important role in bringing green revolution; yields have plateaued to a large extent in many key agricultural crops. A lot in the past depended on the availability of naturally occurring useful genes, some of which might have come from wild relatives through rounds of positive selection. At the same time, a few naturally available alleles might have also cropped up through spontaneous mutation over time.

Given the current importance of genome editing, particularly CRISPR/Cas, it is hoped that the target audience will gain from this book and their combined effort will further facilitate the field of CRISPR/Cas and consumer acceptance of GE products in agriculture. It is imperative that the public must be informed, illustrating, the similarities and differences between genome-edited crops from conventionally bred

crops, to increase acceptance. Public outreach is also one of the objectives of this book.

We hope a bit of illustration on the contents will enhance the book's appeal to the audience. Firstly, the major objective of this volume is to introduce the topic to genome editing, editing types and focuses mostly on the popular CRISPR/Cas technique. The CRISPR/Cas field is vast and almost daily new findings are reported. We have tried our best to include as much information as possible to keep the contents up-to-date. However, it is far from complete by all means. The book begins with a journey on CRISPR/Cas through Chap. 1, which is written by the editors themselves, focuses on genome editing, glimpses on the history of genome editing, classification, basic experimental application followed by genome editing in agricultural crops and challenges it faces. Some of the topics have been described in detail in subsequent chapters. Chapter 2 provides detailed insight on the tools used for designing CRISPR/Cas experiments, bioinformatics aspects, which will be very useful for students, faculties and researchers alike. Chapter 3 provides detailed deliberation on food security by emphasising the role of genes attributing to yield, quality, tolerance to abiotic and biotic stress, and accelerated domestication of crops. Then, Chapter 4 takes us through genetic engineering of floricultural crops. Usually, gene editing in floriculture crops takes a back seat compared to other field and vegetable crops. Therefore, it is our endeavour to include the topic specifically in this book. Chapter 5 discusses applications of CRISPR/Cas to combat multiple abiotic stresses in a crop cycle by modifying key genes. In Chap. 6, a description of use of CRISPR/Cas in vegetable crop improvement is elaborated. We hope this will be very useful to the crop improvement community and draw attention about the usefulness of the technology among vegetable breeders. Further, Chap. 7 focuses on CRISPR/Cas application in climate start agriculture. This is particularly important as climate change is seriously threatening crop production, putting human lives at the risk of impending starvation and malnutrition. A brief outline of the classical crop improvement techniques, proof of concept studies and traits influenced by climatic regimes are discussed. Chapter 8 deliberates on the translational research aspect of CRISPR/Cas in plant genomic research using freely available tools. The application of GE in plant breeding is also discussed for achieving robust plant architecture, flowering and fruit maturation. While, Chap. 9, walks us through the regulatory framework and policy decisions which may finally decide the success of CRISPR/Cas9-GE and positively affect consumer sentiments. Importantly, the chapter deliberates on public understanding of the technology and regulatory acceptance perspective from several nations. Therefore, improving the public image is the key for genome-edited crops to succeed. At the same time, it is imperative that timely approval of biotechnology enabled products and robust regulatory mechanisms must exist so as to facilitate the development of a new generation of gene-edited crops by different stakeholders including start-ups working in this arena. The unwarranted steep regulatory burden puts the additional economic burden to the final product, thus preventing public players from bringing genome-edited to market in a time-bound manner and thwarts private players from investing in new technologies. Chapter 10 focuses on field and cereal crop development. Finally, Chap. 11 walks us through the intellectual property landscape involved with the usage of this technology.

Our readers, at times, may observe a bit of overlap in some topics deliberated in individual chapters. We felt, CRISPR/Cas technique cannot be seen in isolation, and some overlap is completely unavoidable in the context of the topic discussed. This repetitive approach will also help to enforce our understanding of concepts and topic from a different view-point, narrated by individual author and their team for the particular chapter. Finally, we conclude by advocating CRISPR/Cas and associated NPBTs could bring hope for the second wave of the green revolution. This combined with the availability of next-generation sequencing data can help us to create designer crops for the future and defray the ill effects of climate change on global food production to a large extent.

Thus, we hope, this book will provide readers a holistic view of CRISPR/Cas technology and its application in crop plants. We thank Mahyco (Maharashtra Hybrid Seeds Company, Jalna, India), for allowing us to take up this book project and look forward to all suggestions from our readers in making future improvements to the edition. Sincere, special thanks to Dr. Chittaranjan Kole, Series editor for his ever encouragement and team Springer who whole-heartedly accepted our proposal to edit a book project on this important topic of CRISPR/Cas. We sincerely thank all contributors spanning several countries and continents for making this project a success. Thus, for all our combined effort, this book project is able to see the light of the day. We would finally like to dedicate this book to our beloved founder Chairman, Late Dr. B. R. Barwale, World Food Prize Laureate, who was always a strong proponent for use of new plant breeding technologies in modern Agriculture for ultimately improving the livelihood of millions of farmers in India and worldwide.

We wish everyone a happy read!

Jalna, India

Anjanabha Bhattacharya
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Aims and Scope

CRISPR/Cas genome editing (GE) which is referred as new plant breeding technologies (NBTs), can be used as an useful tool for creating improved crop plants. GE involves precise alteration of gene sequences without unwarranted effects in crops. Though traditional breeding has played an important role in bringing green revolution, yields have plateaued to a large extent in many key agricultural crops. NBTs bring hope for a second wave of green revolution. This is particularly important as climate change is seriously threatening crop production, putting human lives at the risk of impending starvation and malnutrition. Therefore, this book aims to introduce readers to GE, various concepts and tools used in GE, and discuss its proven use in modifying agricultural traits in key crops. We will discuss IP (intellectual property) scenario, which is miraged with over-lapping patents and conflicts. Application of CRISPR technology in translational research is also discussed. Lastly, regulatory framework and policy decisions are discussed in light of commercialization of gene edited crops. Thus, this book will provide readers a holistic view of CRISPR/Cas technology and its application in crop plants.

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Abbreviations

AAAS	American Association for the Advancement of Science
AAV	Adenovirus
ABA	Abscisic acid
ABE	Adenine base editor
ACC	1-aminocyclopropane-1-carboxylic acid
ACS	ACC synthase
AFLP	Amplified fragment length polymorphism
ALS	Acetolactate synthase
AOP	Apomictic offspring producer
BADH2	Betaine aldehyde dehydrogenase
Bbm	Baby boom
bp	Base pair
Bt	Bacillus thuringiensis
CAD	Cinnamyl alcohol dehydrogenase
CaMV	Cauliflower mosaic virus
Cas	CRISPR associated protein
CBE	Cytidine base editor
CCD4	Carotenoid cleavage dioxygenase 4
CCD7	Carotenoid cleavage dioxygenase 7
CHLI1	Magnesium-chelatase subunit I
CKX2	Cytokinin oxidase or dehydrogenase
ClCuV	Cotton leaf curl virus
CRISPR	Clustered Regularly Interspersed Short Palindromic Repeat
CRISPRi/a	CRISPR inhibition or activation
CRISTO	Carotenoid isomerise
CSIT	Critical Sterility Inducing Temperature
CTR1	CONSTITUTIVE TRIPLE RESPONSE 1
CVYV	Cucumber vein yellowing virus
dCas9	Deactivated/dead Cas9
DEP1	Dense and Erect Panicle
DMR	Downy mildew resistance
DNA	Deoxyribonucleic acid

DREB2	Dehydration responsive element-binding protein 2
DSB	Double-strand break
eIF4G	Translation Initiation Factor 4 Gamma gene
EIN2	ETHYLENE INSENSITIVE2
ENGase	Endo-N-acetylb-D-glucosaminidase
EPO	European Patent Office
EPSPS	5-enolpyruvylshikimate-3-phosphate
ERFs	ETHYLENE RESPONSEFACTORs
EU	European Union
FAD	Fatty acid desaturase
FAO	Food and Agriculture Organisation
GBSS	Granule-bound starch synthase
GE	Genome Editing
GEEN	Genome Editing with Engineered Nucleases
Ghd7	Grains Height Date 7
GM	Genetic modification
GMO	Genetically Modified Organism
Gn1a	Grain Number 1a
GS3	Grain size 3
Gus	ß-glucuronidase
GVR	Geminivirus Replicon
GW	Grain Width
GW2	Grain weight 2
HAL3	Halo tolerance protein
HDR	Homology-Directed Repair
HD-Zip	Homeodomain-leucine zipper protein
HR	Homologous recombination
HSP	Heat shock protein
Htd1	High tillering date 1
IAEA	International Atomic Energy Agency
IPA1	Ideal Plant Architecture 1
IPK	Inositol phosphate kinase
ISSR	Inter simple sequence repeats
KO	Knock out
LOB1	LATERAL ORGAN BOUNDARIES 1
MAPK3	Mitogen active protein kinase 3
MAPKs	Mitogen-activated protein kinases
MAS	Marker-assisted selection
miRNAs	MicroRNAs
MIT	Massachusetts Institute of Technology
MLO	Mildew Resistance Locus
mRNA	Messenger RNA
MS	Murashige and Skoog
NAA	Napthalene acetic acid
NBTs	New breeding techniques

NCED 1	Phaiustankervilliae9-cis-epoxycarotenoid dioxygenase NCED1
NGS	Next-Generation Sequencing
NHEJ	Non-homologous End Joining
NTWG	New Techniques Working Group
ODM	Oligonucleotide Directed Mutagenesis
OsANN3	rice annexin3 gene
OsDERF1	Drought-responsive ERF gene
OsPMS	Protein mismatch repair
OSR	Organ Size Regulation
P5CS	Pyrroline-5-Carboxylate Synthase
PAM	Protospacer-adjacent motif
PB	Precision breeding
PDR6	Pleiotropic drug resistance 6
PDS	Phytoene desaturase
PEG	polyethylene glycol
PPO	Polyphenol oxidase
PPRs	Pentatricopeptide repeat proteins
PRSV-W	Papaya ringspot mosaic virus type-W
PTAB	Patent Trial and Appeal Board
QTL	Quantative trait loci
RAPD	Random amplified polymorphic DNA
RK2	SNF 1-RELATED PROTEIN KINASE 2
RNAi	interference RNA
RNPs	Ribonucleoproteins
ROS	Reactive oxygen species
RR22	*Oryza sativa* Response Regulator 22
RTBV	Rice tungro bacilliform virus
RTSV	Rice tungro spherical virus
SAGs	Senescence-Associated Genes
SAM	S-adenosyl-L-methionine
SBA	Swedish Board of Agriculture
SBEs	Starch Branching Enzyme
SCR	SCARECROW
SDN	Site-Directed Nuclease
SDNs	Site-directed nucleases
sgRNAs	single guide RNAs
SHR	SHORT-ROOT
SKC1	Shoot K^+ Concentration 1
SlAGL6	Slagamous like 6
SLAGO7	SLARGONAUTE
SNAC2	Stress-responsive NAC gene
SpCas9	*Streptococcus pyogenes*
SPL16	Squamosa Promoter Binding Protein like 16
SSR	Simple sequence repeat
TALEN	Transcriptional Activator-Like Effector Nuclease

TB1	Teosinte Branched 1
TGMS	Thermo-sensitive genetic male sterility
TGW6	Thousand-grain weight 6
tracrRNAs	trans-activating crRNAs
TSS	Transcription Start Site
TT4	TRANSPARENT TESTA4
TuMV	Turnip mosaic virus
UCB	University of California, Berkley
USPTO	United State Patent and Trademark Office
VIGS	Virus-induced gene silencing
Wus 2	Wuschel 2
ZFN	Zinc Finger Nuclease
ZYMV	Zucchini yellow mosaic virus

Chapter 1
Introduction to Genome Editing Techniques: Implications in Modern Agriculture

Anjanabha Bhattacharya, Vilas Parkhi, and Bharat Char

Abstract Climate change accompanied by global warming is happening at an ever alarming pace and is here to stay. Therefore, food production challenges to feed our ever-burgeoning population, which is expected to rise to 9.7 billion by 2050, will remain a steep challenge. Conventional breeding which has created a wave for the first green revolution may not come to our rescue this time entirely alone, in meeting the herculean goal. Therefore, for the second green revolution, agricultural biotechnology-based tools including precision breeding have to play a critical role. For genome editing (GE) induced precision breeding to succeed strong political, social and cultural support is needed. Changing public perception by providing complete information about genome editing will boost consumer confidence and encourage the consumption of GE food crops. Among many GE technologies available, CRISPR/Cas (**C**lustered **R**egularly **I**nterspersed **S**hort **P**alindromic **R**epeats and **Cas** for CRISPR associated proteins), is becoming a tool of choice, due to its simplicity, versatility, scalability and lower cost when compared to other biotechnological tools. Improved versions of CRISPR/Cas-GE, such as base editing are coming to the centre stage and many more technological breakthroughs are expected in the near future. Random mutagenesis based genome editing through physical irradiation and chemically induced, has been accepted all over the globe and it is, therefore, expected that CRISPR/Cas genome editing will be perceived positively. This combined with the availability of next-generation sequencing data can help us to create designer crops for the future and defray the ill effects of climate change on global food production to a large extent.

Keywords Genome editing (GE) · CRISPR/Cas9 · Base editing · Agricultural crops

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1.1 Introduction

Conventional crop improvement is a time-consuming process, which is an interplay of random permutation and combinations to ultimately achieve cultivars with improved yield, biotic, abiotic resistance and quality traits among other economical traits in an era of rapid climate change. However, domestication of crop plants for the last hundred years has resulted in selecting germplasms with narrow genetic base and domestication for predominantly yield traits (Ross-Ibarra et al. 2007; Zhang et al. 2017a, b, Vaughan et al. 2017; Smykal et al. 2018). This has resulted in stagnating yield curve barrier which is difficult to break by conventional breeding techniques alone. This has led to heightening focus on other biotechnology-based interventions including precision breeding (PB) or new breeding techniques (NBTs) (Aglawe et al. 2008). NBTs are broadly collection of techniques which can be efficiently used for crop improvement. Broadly, these include all types of genome editing techniques, genomic assisted tools, gene methylation, *Agro*-infiltration, reverse breeding, oligonucleotide-directed mutagenesis (ODM), single-stranded nucleotide directed editing, SSDN and other molecular biology tools (Schaart et al. 2016; Yoshmi et al. 2016; Mohanta et al. 2017). Oerke (2006) predicted global loss in crop productivity is around 34%, though by some recent estimates is close to 40% (website 1). This might make a lofty goal to feed the World's ever-burgeoning population, which is expected to rise to 9.7 billion by 2050 (website 2). Therefore, it is expected that trait developed through NPBTs can deliver and meet consumer demand. GE (genome-edited) crops can result in the development of traits which, otherwise could be achieved, in only selected cases, by conventional breeding, over several years. While for other traits, conventional breeding may not suffice. Thus, GE helps to meet present demand in a much shorter time period (Duensing et al. 2018). To make our point in favour of precise GE, random mutagenesis/random genome editing in agricultural crops has resulted in the release of more than 2500 cultivars in 175 plant species, which shows that random mutagenesis-based genome editing through irradiation and chemically induced, has been accepted all over the globe (website 3—FAO/IAEA FAO/IAEA 2014; Songstad et al. 2017) and it is expected that present approach of using CRISPR/Cas9 for genome editing will be perceived in a more positive way because of its precise nature. As pointed out in a paper by Wolter and Puchta (2017) that if the plant has one to a few nucleotide change and is transgene-free, it cannot be differentiated experimentally from the one which originated by other means of mutagenesis. For precision breeding to succeed strong political, social and cultural support is needed. Changing public perception in European and other non-GM markets by providing complete information about genome-edited products and cultivating confidence will ultimately decide the future of these new generations of biotechnology-assisted products for crop improvement. Presently, there is a broad spectrum of technologies, that is conventional breeding to GM and now GE, in use for improving agriculture, each of these has already drawn heavy investment from stakeholders. Therefore, not only scientific basis but political, social, economic

consideration and institutional will are needed to capture the usefulness of such technologies. At present, there is a contrasting difference in view-point between European and American consumers in relation to consumption of biotech derived crops. While the American consumer has embraced GM for decades, EU nations have adopted a cautious approach (please see, Lassoued et al. 2018 for detailed results of the social survey on agriculture biotechnology crops). Therefore, improving the public image is the key for genome-edited crops to succeed.

In this chapter, the term CRISPR/Cas includes all variants of Cas including Cas 1, Cas 3, Cas 4, Cas9, Cpf1, Csm, Cas 13. Further, our focus in the present book chapter will be primarily on CRISPR/Cas technology among other GE techniques. Therefore, detailed deliberation will be done involving, CRISPR/Cas technology, mostly due to its wider acceptability, ease of design, scalability, simplicity, speed and overall low cost. While a brief overview will cover other genome editing options available to us, to develop better agricultural crop traits. There are four types of edits which are possible by employing GE, namely, knock out or deactivate a gene, activate or overexpress, repress and precisely alter gene including targeted chromosome rearrangements (Choi and Meyerson 2014). The outcome of GE could result in homozygous, heterozygous, biallelic and mosaic mutations. Broadly, genome editing is divided into four classes according to their chronological order of discovery. Initially, (1) Meganucleases (MNs) or customized homing nucleases were used in gene editing followed by (2) Zinc finger nucleases (ZNFs), (3) TALEN and (4) CRISPR/Cas (Fig. 1.1a–d). While MNs, ZNFs and TALENs are protein-based, CRISPR/Cas is an RNA–protein

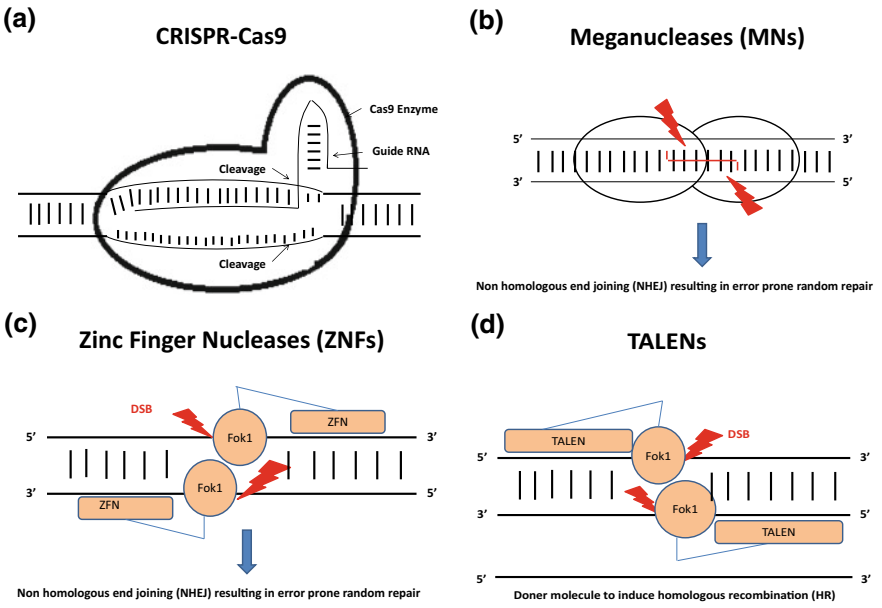

Fig. 1.1 Four class of GE tools

hybrid system. We will describe each of these technologies one by one starting with CRISPR/Cas. Essentially, GE mainly focuses on precise genetic manipulation of a target sequence, but many other application including transcriptional regulation, genomic screens, imaging and diagnostics is also possible.

1.2 History and Origin of CRISPR/Cas

CRISPR stands for **C**lustered **R**egularly **Interspersed S**hort **P**alindromic **R**epeats and **Cas** for CRISPR associated proteins. This is basically, an acquired immune system of bacteria and archaea against invading viruses (Barrangou and Marraffini 2014). Bacteria are in constant war with invading viral particles. Thus, in order for bacteria to survive a plethora of viral attack, bacteria developed an innate immune system, termed as CRISPR/Cas, a two-component system. The system degrades foreign DNA that re-enters the cell. Once a virus attacks bacteria, built-in CRISPR system copies segment of the repeats of the viral genome which are few base pairs long of extrachromosomal origin, separated by non-coding sequences known as spacer sequences (Bolotin et al. 2005) adjacent to PAM (Protospacer Activating Motifs) (Karginov and Hannon 2010). When the second round of attack takes place, the CRISPR/Cas system is activated and degrades the viral genome. In short, CRISPR/Cas is remembered and destroys the system. The Cas library not only maintains an array of records of invading viral signature but also helps to destroy upon re-infection. Previously, it is well known that phage infection is a serious problem in dairy business (website 4) and it became increasingly challenging to maintain an original pure culture of bacterial strain for a wide basket of products offered to consumers. Thus, yoghurt makers have been unknowingly relied on CRISPR/Cas system to fight bacteriophage attacks (Garneau and Moineaus 2011; Marco et al. 2012). This relationship between CRISPR sequences and bacteriophage was elucidated and is elaborated in Hille and Charpentier (2016). The length of CRISPR sequences relies on the rate of active spacer acquisition, depending on viral infection pressure. However, the significance of CRISPR/Cas system was not elucidated till researchers discovered that CRISPR system acts like RNAi (reviewed in Shabbir et al. 2016). The history of CRISPR goes back to 1987, when Japanese scientist, Yoshizumi Ishino, from Osaka University, accidentally discovered repeated sequences in *E. Coli* (Ishino et al. 2018), though the role of repeated sequences was not known then. Soon afterwards, in 1993, researchers in Netherland discovered direct repeats in bacteria. Then, researchers in the University of Alicente, Spain discovered repeat sequences in *Archea* (Mojica et al. 1995; Mojica and Rodriguez-Valera 2016). Practical application of CRISPR/Cas in prokaryotes and eukaryotes were developed (refer to Hsu et al. 2015), thus, CRISPR/Cas was designated as the breakthrough technology of the year, 2015, by the American Association for the Advancement of Science, AAAS (website 5). Truly, CRISPR/Cas is a disruptive technology involved in rewriting the genetic code. Some believe that CRISPR/Cas technology is superior to RNAi (interference RNA, a gene silencing technique), as it can create knock out (KO) while RNAi

cannot fully suppress gene activity under a varying array of environmental conditions (Boettcher and McManus 2015; Unniyampurath et al. 2016; Arora and Narula 2017). Here, it must be mentioned that while bacteria have developed CRISPR/Cas system to defend themselves against phage/ virus, the phage itself has counter developed anti-CRISPR/Cas strategy. This involves phage mutating CRISPR library thereby evading destruction or have developed protein which prevents Cas binding. Anti-CRISPRs are also common in mobile genetic elements. The anti-CRIPR proteins are small enough to pass through the nuclear pores (Pawluk et al. 2016). However, anti-CRISPR strategies are few and far in between and CRISPR/Cas system is likely to dominate the field of genome editing (see Pawluk et al. 2018).

1.3 Mode of Action of CRISPR/Cas System

CRISPR/Cas is an RNA-guided nuclease which edits the DNA sequence. Generally, U3/U6 (RNA pol III promoter) is used to drive SgRNA, while CaMV35S and ubiquitin promoter is used to drive Cas9. Both gRNA and Cas must be expressed in the cell at the same time for the editing to take place. The most commonly studied system is CRISPR/Cas9 from *Streptococcus pyrogens* (type-II) and Cpf1 (type V). It must be noted that Cas9 is better than Cas type-I, which employ multiple subunits rather than a single one. The **CRISPR/Cas** system comprises of monomeric DNA non-specific endonuclease (Cas 9) and customizable single guide RNA (synthetic guide RNA). The guide RNA is a two-component system and contains CRISPR RNA (crRNA) and transactivating (trRNA, Deltcheva et al. 2011), which direct Cas9 protein to the target sequence and results in site-specific cut, generally in the vicinity of 3–4 bp upstream of PAM (protospacer adjacent motif) site. The PAM is necessary for Cas9 binding to the DNA sequence, adjacent to the sequence sgRNA represent (Jinek et al. 2012). For the formation of mature guides from pre-cr RNA to happen, processing and transcription take place from the DNA sequence. For clarity, we will use the term g or Sg-RNA for the synthetic fusion of cr-RNA and trancr-RNA, that is target sequence with scaffold sequence for ease of understanding. This guide RNA facilitates editing and is easy to design. Therefore, guides are essential as well as the star feature of the CRISPR/Cas system. Editing takes place by the formation of RNA–DNA complex, followed a repair mechanism that uses template/donor DNA, causing DNA cleavage by Cas9 complex (Huai et al. 2017; Sundaresan et al. 2017) which is subsequently repaired by endogenous repair mechanism. In order to increase the efficiency of editing, interaction of non-target strand, positively charged groove of the Cas complex and gRNA with target strand should be optimized (Guha et al. 2017). This can be achieved by weakening the bond in the Cas complex, thus increasing specificity. The DNA repair is of three types, that is error prone repair of DNA occurs by NHEJ (nonhomologous end joining, Schiml et al. 2014) and precise edits which, happens by DSB (double-stranded break in both leading and lagging strand, Anders et al. 2014) induced by homologous directed repair (HDR). CRISPR/Cas induced HR (homologous recombination) may involve biolistics, as this helps to increase

copy number and facilitate HR process. The third one is micro-homology mediated end-joining (MMEJ) is a repair process involving very short homology regions (5–25 bp) flanking DSB (for details, see, Seol et al. 2018). The system is limited by the availability of the PAM region in the gene of interest. HDR mediated insertions have been achieved by *Agrobacterium*-mediated transformation. Cas9 has three domains, the larger one being REC connected to the smaller one (RuvC) and the third HNH-nuclease domain, which results in a positively charged groove and binds to PAM sequence. An added advantage of CRISPR/Cas9 is that it can be used to simultaneously edit several genes (Ma et al. 2015; Li et al. 2018a, b, c; Sekine et al. 2018). CRISPR/Cas system themselves do not have any special preference for coding and non-coding sequences (Canver et al. 2017). Another Cas protein, Cas 12a (Cpf1) is derived from *Provoltella*, *Francisella*-1 (Zetsche et al. 2015) with the recognition, T rich, TTTN PAM site. Cpf1-gRNA is much smaller, 42nt long compared with Cas9 which is 92 bp long and Cpf1 has high multiplexing capability compared to Cas 9 (Li et al. 2018a, b, c). Cpf1 locks HNH endonuclease domain and needs one RNA instead of both cr- and transcr-RNA for editing. Cpf1 is much smaller than Cas9, is preferably used in HDR and gene insertion or replacement. Cpf1 induces sticky overhangs of 5 bp, 18–23 bp away from PAM site whereas Cas9 induces blunt cut, 3 bp upstream of PAM site (refer to Deng et al. 2018). Cpf1 is better at editing non-dividing cells. Since, Cpf1 results in staggered cut way from PAM site, it is retained for editing, sometimes referred as second chance editing, facilitating HDR repair (for details refer Zetche et al. 2015). However, it should be ensured that once HDR is complete, the homologous sequence or repair template must have an inactive PAM site to prevent the possibility of subsequent editing. Cpf1 can facilitate self-cleavage activity involving multiple genes and therefore is better in editing genes than Cas9 (Li et al. 2018a, b, c). Cpf1 lacks HNH therefore, conversion to nickase is not possible. Three other orthologs of Cpf1 are available from *Francisella novicida* (FnCas12a), *Lachnospiraceae bacterium* ND 2006, *Acidaminococcus* sp BV3L6 (Verwaal et al. 2017). Dead Cas9, can be used to develop inactive versions of the target gene (CRISPRi, Dominguez et al. 2016) which can be used in epigenomic editing (Qi et al. 2013; Larson et al. 2013). The potential application of dCas9 may include, modulating the expression pattern of gene, using a modified version of Cas9. Another point to note is that dead Cas versions used for activation or repression do not cause off-target editing problems. The development of Cas9nickase results in cleavage of only one strand of DNA and thus lowering the frequency of off-target editing (Sander and Joung 2014). Nickase is appropriate in directing sequence tags and mutations employing GE studies. Enhanced eSpCas9 and eSpCas9-HF1 are available which reduces Cas activity and at the same time increasing sensitivity to specific edits in the target gene (Slaymaker et al. 2016). Engineered versions of Cas are now available for precision editing. Further, the availability of multiple Cas9 orthologs facilitates individual Cas9s to be used together in one experiment (Tycko et al. 2017). Alternatively, there is another approach to deliver CRISPR/Cas into the cell, which is based on the protein-RNA complex, thereby bypassing transcription and translation associated with DNA editing. The alternative technique is known as