Chapter 14
CRISPR/Cas13: A Novel and Emerging Tool for RNA Editing in Plants

Deepu Pandita, Chandra Obul Reddy Puli, and Sudhakar Reddy Palakolanu

Abstract Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR) and CRISPR-associated protein (Cas) act as an adaptive immune system against invading nucleic acids and bacteriophages in bacteria and archaea. Based on the constitution of effector protein, CRISPR/Cas is broadly divided into multiple types and subtypes. Among these, type VI CRISPR/Cas system is of special attention with four subtypes, namely, VI-A, VI-B, VI-C, and VI-D, and are believed to have evolutionary origin from transposons. These subtypes exhibit variations in structural architecture and mechanism and have diverse Cas13a (C2c2), Cas13b1 (C2c6), Cas13b2 (C2c6), Cas13c (C2c7) and Cas13d effector proteins. CRISPR/Cas13 ribonuclease processes pre-crRNA to mature crRNA which targets and knockdown single-stranded RNA of phage genome during viral interference. The high specificity RNA guiding and RNA-targeting capacity of this protein enables to fuse with several effector molecules, opening new avenues in the field of Cas13-mediated RNA targeting, tracking, and editing. CRISPR/Cas13 has a unique feature of targeting RNAs including plants, so it can be used as a new tool for engineering interference against plant pathogens including RNA viruses, with better specificity and for other RNA modifications in plants. Fluorescent probe-tagged deactivated programmable Cas13 proteins could be used as an alternative tool for in *vitro RNA* studies. The engineered Cas13 can also be used for programmable RNA editing. The high target specificity, low cost, and user-friendly operation of CRISPR/Cas13 make this an effective tool for several RNA-based research studies and applications. Therefore, the focus of this chapter is upon classification of CRISPR/Cas system, structural and functional diversity of type VI CRISPR/Cas system including its discovery and origin, mechanism, and role of Cas13 in RNA editing of plants.

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14.1 Introduction

The limited cultivable land and ever-changing climate pose severe challenges to global food security. The steady rise of human population (reaching nine billion by 2050) requires almost 100–110% more food grains over the present levels (Tilman et al. 2011). This has to be achieved from the limited fertile land, water, fertilizers, and pesticides and under the threat of climate change. The traditional breeding and marker-assisted breeding largely depends on natural genetic variations in the germplasm. Spontaneous mutations cause natural genetic variations during the evolution, and domestication of a crop species, which serves the need to some extent. However, for all the major crops, genetic information is fixed and they tend to lose their genetic variability due to the practice of directed evolution through breeding for several years (Jung et al. 2018; Pacher and Puchta 2017). Also, traditional breeding is a time-consuming process, and not in a position to meet the demands of sufficient food supply for the growing population. In the past five decades, induced mutations either by chemicals or irradiation have been expansively employed to generate new allelic variations in the plants. Induced mutations created genetic variations in several monogenic and in some cases in quantitative traits also. However, the major problem in mutational breeding is that the mutations are not targeted, occur throughout the genome, and can be detected only through extensive phenotypic screening of a huge population. Later on, TILLING technology-enabled detection of phenotypes specific to targeted genes. Nevertheless, generation of huge number of offsprings, unintended mutations, polyploidy nature of crop species are major hurdles for mutational breeding (Tadele 2016). In the recent past, crop breeding has been enriched and Genetically Modified (GM) crops were developed with the advancements in gene cloning and transformation of selected plant species. Several GM crops have been developed for various important traits by overcoming cross-species barriers. However, due to the regulations, public concern, and fears, only a few developing and developed countries accepted GM crops for cultivation (Prado et al. 2014).

Therefore, rapid and target-specific technologies are required for the creation of novel alleles across the genomes without leaving any leftover DNA or RNA or protein of foreign origin. Several groups focused on the development of tools for target gene-specific homologous recombination (HR), by the introduction of double-strand breaks (DSBs). In this approach target sequence-specific endonucleases were used to create repairable breaks in the dsDNA, which further forced to adopt either non-homologous end joining (NHEJ) or homology-directed repair (HDR) (Pardo et al. 2009). Advancement in recombinant DNA technology and with the continuous efforts of scientists, novel synthetic tools were developed to address the issue of

precise gene editing in plants. In the year 1996, a synthetic, chimeric, sequence-specific cleavage protein complex "zinc finger nucleases (ZFNs)" was developed. It consists of two parts: zinc finger protein domain for recognizing and binding to specific DNA sequences and, the *Fok*I endonuclease domain to cleave DNA precisely at a defined region (Kim et al. 1996). Soon ZFNs became a popular method for site-specific gene editing both in the model and crop plants (Wright et al. 2005; Lloyd et al. 2005; Maeder et al. 2008; Tovkach et al. 2009; de Pater et al. 2009; Townsend et al. 2009; Cai et al. 2009; Shukla et al. 2009; Zhang et al. 2010; Osakabe et al. 2010; Weinthal et al. 2010; Petolino et al. 2010). However, often low target efficiency of ZFNs leads to off-targets and also designing, assembling of arrays in a construct is laborious and expensive, thus limiting the applications of ZFNs (Maeder et al. 2008; De Francesco 2012).

The quest in search of efficient DNA binding proteins for editing of genomes; identified "transcription activator-like effectors (TALE)" as a substitute for ZFNs in the year 2010. Synthetic TALEs have a DNA binding domain which in turn comprise of a vital repeat domain with 33–34 highly repeated conserved amino acid sequences with a variation in 12th and 13th amino acids called Repeat Variable Diresidue (RVD) (Boch and Bonas 2010). Each TALE protein identifies a single nucleotide on the DNA strand. Similar to ZFNs, TALE domains are also fused with *Fok*I nuclease and are directed in a head-to-head tandem to trigger DSB in the desired target nucleotide sequence (Voytas 2013). TALENs are easier to engineer, therefore, a huge resource of engineered TALEs are available and novel techniques such as Golden Gate and Platinum Gate made easier and less time to assemble them in a construct, thus TALENs became more favorable gene editing (GE) nucleases compared to ZFNs (Gupta and Kiran 2014; Zhang et al. 2013a, b). So far, endogenous genes of several models and crop plants have been successfully targeted by TALENs and mutations were created (Shan et al. 2013a; Qi et al. 2013; Wendt et al. 2013; Haun et al. 2014; Wang et al. 2014; Gurushidze et al. 2014; Lor et al. 2014; Sprink et al. 2015; Kazama et al. 2019). TALENs are not only used for the generation of mutations in the desired sites but also for gene regulation by combining DNA binding domain with activator and repressors (Mahfouz and Li 2011; Gao et al. 2014).

The most recent addition to the GE toolbox was Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR), in which rather than DNA binding protein, an RNA molecule appends to the target DNA strand on a complementary basis and further Cas protein causes the cleavage at the target site (Wiedenheft et al. 2012; Sorek et al. 2013), which makes this system as simple and user-friendly over the ZFNs and TALENs. The CRISPR/Cas9 system is an adaptive immune response system of bacterial and archaeal systems to protect against the invading viruses (Marraffini and Sontheimer 2008; Horvath and Barrangou 2010). The engineered CRISPR/Cas9 system consists of two components: a short synthetic RNA molecule (single guide RNA) complementary to upstream of an NGG trinucleotide PAM (Protospacer Adjacent Motif) of a target DNA sequence fused with a crRNA and a fixed trans-activating crRNA and another component a DNA nuclease called Cas9 (Jinek et al. 2014; Jiang and Doudna 2015). Upon binding to a target DNA sequence, the inactive Cas9

forms two active nuclease domains which further trigger the DSB in the upstream region of PAM (Jinek et al. 2012). The CRISPR/Cas9 nucleases may be optimized to target any specific DNA sequence by simply designing the gRNA sequence. Hence, CRISPR/Cas9 nucleases have been widely used in diversified organisms for targeted GE (Hsu et al. 2014; Doudna and Charpentier 2014). The CRISPR/Cas9 system is functional in plant systems for various applications and the mutations generated are inheritable to the subsequent generations and follow Mendelian inheritance (Feng et al. 2013; Jiang et al. 2013; Li et al. 2013; Mao et al. 2013; Miao et al. 2013; Nekrasov et al. 2013; Shan et al. 2013b; Upadhyay et al. 2013; Schiml et al. 2014; Feng et al. 2014; Jiang et al. 2014; Xie and Yang 2013; Xing et al. 2014; Zhou et al. 2014). However, the number of off-target cleavages limits the applicability of CRISPR/Cas9 technology. Several modifications have been made in Cas9 enzymes such as an increase in the protospacer adjacent length and identification of novel Cas9 enzymes with unique and expanded PAM sequences from various bacterial species improved the target specificity (Ran et al. 2015; Lee et al. 2016; Hu et al. 2016; Zetsche et al. 2017). Additionally, catalytically deactivated Cas9 (dCas9) derivative CRISPR/dCas9 can be attached with regulatory proteins such as activators, repressors, reporter genes and used as a basic research tool for crop improvement (Lowder et al. 2017a, b; Zhang et al. 2016; Dreissig et al. 2017; Zezulin and Musunuru 2018; Gjaltema and Schulz 2018; Hilton et al. 2015; Guo et al. 2015; Xue and Acar 2018; Liang et al. 2017; Veillet et al. 2019; Woo et al. 2015; Moradpour and Abdulah 2020).

The desired nucleotide sequence can be incorporated in the plants through DSB-induced HR in the process of GE. However, this process is laborious and inefficient. To overcome the pitfalls, several Cas-derived base editing strategies were developed with the fusion of cytidine deaminases or adenosine deaminases to Cas9 or dCAS9 effector for the conversion of C/G to T/A without any DSB. Recently, computational analysis of genomes of bacteria and archaea led to the discovery of Cas13 (previously C2c2) protein, which has dual eukaryotic and prokaryotic nucleotide-binding RNAase domains, thus can cleave RNA transcripts precisely in a nucleotide base-specific manner; extending the editing facility to RNA also (Abudayyeh et al. 2016; Shmakov et al. 2015). Additional studies identified that Cas13 was also able to carry deamination of adenosine to inosine (A to I) through Adenosine Deaminase 2 (ADR2) in a programmable manner *i.e.*, "RNA Editing for Programmable A to I Replacement" (REPAIR) (Cox et al. 2017). Moreover, heterologous expression of LwaCas13a (a more active orthologue of Cas13 from *Leptotrichia wadei*), in mammalian and plant cells knocked down the endogenous and reporter transcripts (Abudayyeh et al. 2017). Similarly, transient and stable heterologous overexpression of LshCas13a in *Nicotiana benthamiana* cell lines exhibited modest resistance to an RNA virus Turnip Mosaic Virus (TuMV) by degrading the virus RNA (Aman et al. 2018a). These studies open up a new avenue for the use of RNA editing in the fields of basic research and crop improvement.

14.2 CRISPR/Cas System

14.2.1 Discovery and Mechanism

Modern agriculture revolution has started with the great advantage of tools such as whole-genome sequencing, resequencing of the genomes, and new breeding technologies (NBTs), for example, genome editing. Genome edited plants are differentiated from genetically engineered plants in terms of integration, precision, and efficiency. The GE tools such as (ZFNs, TALENs, and CRISPRs) have been explored to achieve inherent and efficient site-directed mutagenesis in a predefined manner. All site-directed nucleases break the target DNA sequence at specific sites and utilize the plant's natural DNA repair mechanism to repair the DSBs through either HDR or NHEJ, resulting in the intended sequence alterations ranging from point mutation to large insertions or deletions (INDELs) at predefined sites in the target genomes. However, ZFNs and TALENs are more expensive, complex, and laborious to adopt. Henceforth, CRISPR/Cas-based tools became game-changer, user-friendly, and more efficient tools in performing GE activities in achieving the precisely targeted mutagenesis in animals and plants without any negative impact on the native plant phenotype.

CRISPR/Cas tool needs a short (~20 bp) guide RNA (gRNA) sequence to identify the target location by Watson–Crick base-pairing. Cas nuclease enzyme recognizes PAM sequence and cleaves at a site 2–3 bp away from it (Jinek et al. 2014; Zetsche et al. 2015). The endonuclease action of Cas protein can prompt quality change by cutting the target DNA and framing DSBs that lead to DNA repair *in vivo* using natural repairing mechanisms, thus creating modifications in the targeted genome (Lowder et al. 2016; Zhang et al. 2016; Chen et al. 2016). CRISPR/Cas tool was able to overcome the drawbacks of its predecessors, ZFNs, and TALENs, which would have random off-target binding and technical complexity. Due to this, CRISPR/Cas tool gained more popularity over ZFNs and TALENs. Additionally, the comfort of use and enhanced editing efficiency helped to demonstrate it as a potential method for handling a range of genomes, including complex genomes. Cas protein has multiple domains that help in adjustment, taking part in the processing of the pre-crRNA into crRNA, and making DSBs (Mulepati et al. 2015).

14.2.2 Applications of CRISPR/Cas System

Multiplex genome editing utilizing numerous gRNAs to target different genomic locations at the same time has been demonstrated using CRISPR/Cas system. The past few years have witnessed rapid growth in genome-edited crops using CRSPS/Cas tools. In plants, more than 20 crop plant species have utilized the CRISPR/Cas tools for various applications of crop improvement (Ricroch et al. 2017; Jaganathan et al. 2018). The potential use of these methods has been established in many plants such

as *Zea mays* (Shukla et al. 2009), *Nicotiana benthamiana* (Nekrasov et al. 2013), *Oryza sativa*, *Arabidopsis thaliana* (Jiang et al. 2013), and major crops such as maize, wheat (Wang et al. 2014), rice (Feng et al. 2013; Xie and Yang 2013; Zhang et al. 2014), tomato (Bortesi and Fischer 2015; Brooks et al. 2014), and sorghum (Liu et al. 2019; Che et al. 2018; Sander 2019). CRISPR/Cas tool has been efficiently used to introduce climate-related agronomic traits including drought stress (Duan et al. 2016; Shi et al. 2017; Mishra and Zhao 2018), salinity (Zhang et al. 2019a), among others. The application of the CRISPR/Cas tool in disease resistance was deployed to achieve biotic resistance by targeting different genes. Resistance to tungro and blast diseases have been reported recently in rice using CRISPR/Cas tool by targeting *eIF4G* (Macovei et al. 2018) and *OsERF922* genes, respectively (Wang et al. 2016). Oliva et al. (2019) targeted *SWEET* genes using the CRISPR/Cas tool for achieving broad-spectrum resistance to bacterial blight (Oliva et al. 2019).

14.2.3 Classification of CRISPR/Cas System

Alterations in technological advances will affect the advancement of agriculture and associated fields since it will permit fast and efficient genetic modifications in the targeted genomes. CRISPR/Cas tool has been effectively used to modify pathways and key genes to enhance the crop qualities. Most of the research efforts done to date have utilized the Cas9 nuclease for targeted genome editing. Even though, it has promising applications, several concerns over CRISPR/Cas9 technology such as: the large size of the Cas9 molecule, off-target effects, recognition of PAM motifs and effective delivery, and low efficiency via HDR. One possible way is to use efficient Cas variants with fewer off-targets and efficient editing. Also, modified Cas variants show efficient editing with fewer off-targets as demonstrated in various species (Kim et al. 2018; Yin and Qiu 2019). Hence, an alternative to Cas9 protein, variants such as dCas9, CRISPRi, iCas9, nickase79, fCas980, Cpf181, C2C2, 13B, Cpf1, and others, came into the spotlight.

Based on the type of effector protein, the CRISPR system is divided broadly into two Classes 1 and 2 and further based on the sequence conservation and organization it is divided into six types (I–VI) and many subtypes. The main basis for the classification is how the effector molecules associated with target surveillance and defense. In Class 1 system (types I, III, and IV), the effector is composed of multiple proteins while Class 2 system (II, V, and VI) is constituted of a single domain (Koonin et al. 2017). The detailed classification of the CRISPR/Cas system is highlighted in Fig. 14.1.

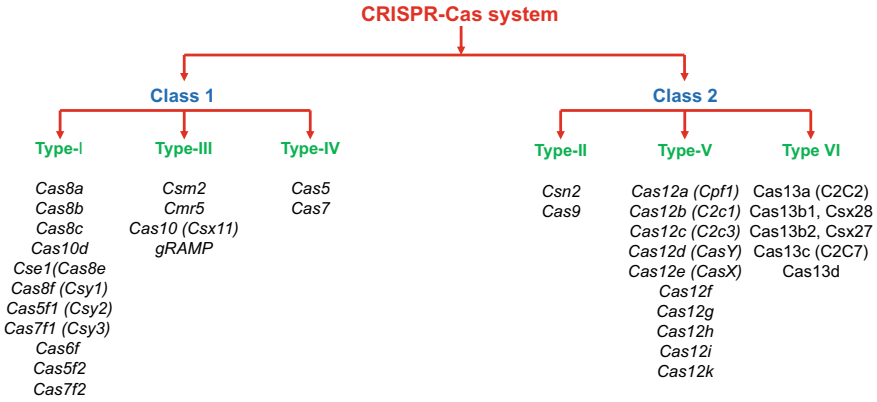

Fig. 14.1 The Classes, types, and subtypes of CRISPR/Cas systems: CRISPR-Cas system is divided into two classes: Class 1 and Class 2 on basis of Cas proteins. Class 1 has a set of effector complexes while class 2 contains a single protein. Each class consists of 3 types: Class 1 system contains type I, type III, and type IV whereas Class 2 system contains type II, type V, and type VI. Each type is further subdivided into various subtypes (Makarova et al. 2020)

Over the past few years, Class 2 system has shown to have multiple flexible applications such as knock-outs, genetic screening, imaging, etc., using different Cas systems such as Cas9, Cas12a/Cpf1, and Cas13 (Tang and Fu 2018). Although Class 1 CRISPR system can target RNA (Kazlauskiene et al. 2017; Niewoehner et al. 2017), type VI system has been identified recently to exclusively target RNA (Abudayyeh et al. 2016; Konermann et al. 2018; Smargon et al. 2017; Yan et al. 2018). Type VI system from Class 2 has the most unusual characteristic feature that targets ssRNA rather than dsDNA (Abudayyeh et al. 2017). Cas13 molecule comprises of a gRNA-directed ribonuclease. This ribonuclease system tends to cleave RNA non-specifically and has four subtypes (Cas13a, Cas13b, Cas13c, and Cas13d) (Cox et al. 2017). Another unique feature of Cas13 is that it has dual HEPN (Higher Eukaryotes and Prokaryotes Nucleotide-binding) nuclease domains, which creates blunt ends during RNA editing (Cox et al. 2017). Recent studies have highlighted that Cas13 has fewer off-target effects and has better precision in GE applications (Konermann et al. 2018). Together, in summary, Cas13 effector molecules have demonstrated higher effectiveness and efficacy compared to its counterparts such as Cas9 in several crop species (Aman et al. 2018a; Zhang et al. 2018c). The detailed mechanism, classification, and applications of Cas13 systems are explained in detail in the following sections.

14.3 CRISPR/Cas Type VI System (Cas13)

14.3.1 Discovery

Type VI CRISPR/Cas system includes subtypes VI-A, VI-B, VI-C, and VI-D, featuring Cas proteins with varied sizes and DNA sequence. The type VI CRISPR/Cas system has a modest structure with two HEPN domains and needs a single Cas13 protein and crRNA molecule for action (Smargon et al. 2017; Shmakov et al. 2015). Shmakov and co-workers (2015) developed a computational pipeline for scrutiny of the entire microbial genome sequences from the National Centre for Biotechnology Information—whole genome shotgun (WGS) database based on the incidence of Cas1 for identifying the uncategorized candidate Class 2 CRISPR loci. CRISPR/Cas loci contain the utmost conserved Cas1 gene (Makarova et al. 2015), because of which Cas1 was used as a query for identification of the candidate loci. The identified CRISPR candidates were labeled according to the order of discovery. Shmakov and co-workers (2015), with the use of bioinformatics tools, predicted a new Class 2 effector type VI subtype termed Class 2 candidate 2 (C2c2) by using Cas1 as the seed. The C2c2 (meaning Class 2 candidate 2) was detected from 21 bacterial genomes belonging to five chief taxa of Bacteroidetes, Bacilli, α proteobacteria, Fusobacteria, and Clostridia. The C2c2 loci comprise a hefty protein. The initially recognized C2c2 loci included the Cas1 and Cas2 proteins. Succeeding main searches displayed the presence of only the C2c2 locus and CRISPR array. Such structurally partial loci may either produce flawed CRISPR/Cas system transcripts or may act nonautonomously by exploiting the adaptation module present at a far distance in that genome (Majumdar et al. 2015). Shmakov et al. (2015) investigated the function of C2c2 loci. They synthesized locus C2c2 of *Listeria seeligeri serovar* which when expressed in *E. coli* produced CRISPR RNAs with 5′ 29-nt direct repeats and 15–18-nt spacers. C2c2 locus of *Leptotrichia shahii* showed expression in *E. coli* and processed CRISPR array into 44-nt crRNAs. This gene locus of C2c2 comprise of an expected putative tracrRNA (trans-activating crRNA) without any expression. *A. acidoterrestris* putative tracrRNA holds a distinctive CRISPR anti-repeat sequence. Prediction of the potential tracrRNAs for the C2c2 loci was done by probing anti-repeat sequences inside C2c2 locus. The CRISPR/Cas loci in numerous C2c2 systems have degenerated repeated sequences positioned at the promoter-distal terminal of the CRISPR array (Biswas et al. 2014). The putative tracrRNAs were confirmed in four out of 17 C2c2 loci, however, their functional relevance remains to be determined. The protein sequences of C2c2 had two R (N) xxxH motifs with conservation which are typical of HEPN domains (Anantharaman et al. 2013; Grynberg et al. 2003). The C2c2 sequences in the Pfam database were similar to domains of HEPN for putative domains of C2c2 nuclease. The C2c2 sequences exterior to 2 HEPN domains show mixed alpha/beta helical structure lacking a distinct connection to identified

proteins. This uniqueness guarantees that C2c2 belongs to the type VI CRISPR/Cas system (Shmakov et al. 2015). The effector protein having dual HEPN domains is the signature of type VI CRISPR/Cas systems (Makarova et al. 2014; Anantharaman et al. 2013). The first putative type VI effector, C2c2 belonging to Class 2 Type VI CRISPR/Cas system, is now known as CRISPR/Cas13a. Association of HEPN domains with RNase perspective suggested that Cas13a acts as RNA-guided RNase and targets RNA which was later validated experimentally, showing that type VI Cas13a effector possesses a single-strand RNA-targeting capability in RNA bacteriophage MS2 (Abudayyeh et al. 2016). C2c2 protein facilitates interference, pre-crRNA processing (East-Seletsky et al. 2016) and shows a coupler effect of adaptive immunity with programmed cell death which was predicted previously by comparative genomic investigation (Makarova et al. 2012) and mathematical modeling (Iranzo et al. 2015). Afterward, the structure of Cas13a was analyzed (Liu et al. 2017a, b).

Various identified functional CRISPR/Cas systems show nonautonomous nature which lack Cas1 and are dependent upon adaptation modules (Cas1 and Cas2) of additional CRISPR/Cas systems of the genome (Anantharaman et al. 2013; Makarova et al. 2015). Because of this, their detection in previous analyzes based on Cas1, as the seed, was not possible (Shmakov et al. 2015; Makarova et al. 2015). The investigation for CRISPR/Cas loci by using CRISPR repeat arrays as an anchor/seed led to the identification of 13 novel subtypes and five additional tentative subtypes lacking the adaptation module in Class 2 CRISPR/Cas system (Burstein et al. 2017; Shmakov et al. 2017; Koonin et al. 2017; Smargon et al. 2017). This analysis discovered the existence of four distinct putative Class 2 effector subtypes: VI-A, VI-B1, VI-B2, and VI-C in type VI CRISPR/Cas systems, unrevealed in the preceding studies (Shmakov et al. 2015; Makarova et al. 2015). The C2c2-encoding locus was named as subtype VI-A and C2c6—encoding loci were named as subtype VI-B. The classification of type VI into various subtypes emphasized that HEPN domains exist at various locations of Cas13. The RNA-guided VI-B type loci which encode transmembrane domains were separated into VI-B1 and VI-B2 subtype variants of RNA-targeting nature (Shmakov et al. 2017; Smargon et al. 2017). Subtype VI-B is restricted to the phylum Bacteroidetes and Cas13b proteins also show collateral RNase activity. During evolution VI-B1 (membrane-associated RNA-targeting systems) and VI-B2 variants diverged according to the different structural designs of the allied predicted membrane proteins (Shmakov et al. 2017). With an updated bioinformatics pipeline and additional genomics and metagenomics datasets, the Class 2 effector discovery approach widened the net to fish unrevealed effector proteins of Class 2 CRISPR/Cas system (Konermann et al. 2018; Yan et al. 2018). A new subtype VI-D of type VI CRISPR/Cas loci and Cas13d effector protein was identified predominately in *Eubacterium* and *Ruminococcus* (Yan et al. 2018; Konermann et al. 2018). The historical timetable of the detection of type VI RNA-targeting CRISPR/Cas system is shown in (Fig. 14.2).

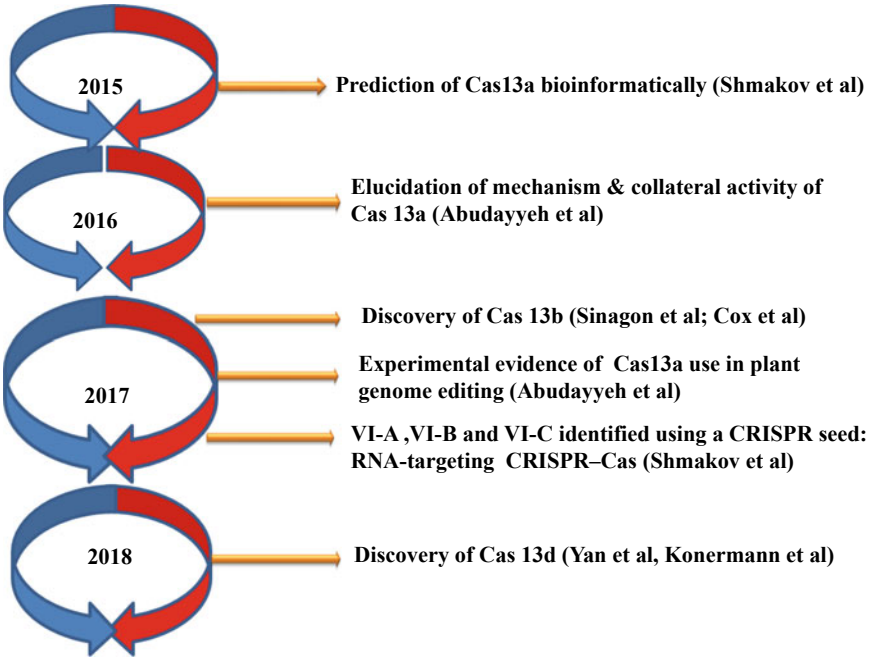

Fig. 14.2 Historical timeline of the discovery of type VI CRISPR/Cas system

14.3.2 Evolutionary Scenario for Type VI CRISPR/Cas Systems

The evolutionary hypothesis states that the origin and integrated evolution of putative Class 2 type VI CRISPR/Cas system have occurred from transposable genetic elements. The Cas 13 translation product is distinct from extra Class 2 protein effectors. Because of that, the identification of type VI CRISPR/Cas system resolves that Class 2 variants have originated independently (Shmakov et al. 2015). The type VI CRISPR/Cas system conscripted one HEPN—protein domain during evolution. This HEPN—protein domain underwent duplication and additional expansion in size. HEPN domains have not been identified in bona fide transposons. HEPN domains show horizontal transfer and are essential to a few transposable genetic elements, for example, toxin–antitoxin units (Anantharaman et al. 2013). There is a probability that Cas13 effector proteins might have originated from ancestral mobile components of HEPN-containing toxins. The origin of ancestral adaptive immunity might

have taken place by the insertion of Cas1-encoding transposon known as casposon besides a locus of innate immunity system (Koonin and Krupovic 2015; Krupovic et al. 2014). The evolutionary steps involved in the origin of type VI CRISPR/Cas system can be summarized as under (Shmakov et al. 2015, 2017):

1. Origin of ancestral adaptive immunity system by fusion of Cas10-like gene with casposon and Cas2-like toxin
2. Origin of ancestral Class 1 system by fission of Cas10-like protein and duplication of RRM domain
3. Origin of ancestral Class type 3 system involving duplication of Cas7
4. Origin of type VI system of Class 2
5. The annexation of HEPN domain protein effector module besides the Cas1 and Cas2 adaptation module (innate immunity protein)
6. Fixation of the functional modules (that is adaptation and effector modules)
7. Replication of HEPN domain protein
8. Auxiliary co-evolution of binary modules of adaptation and effector
9. Acquirement of adaptation module in few type VI CRISPR/Cas systems.

Cas proteins may be categorized as functional effector and adaptation module. The adaptation module contains largely uniform Cas1 and Cas2 proteins which integrate DNA into CRISPR arrays and generates crRNAs. The extremely variable effector module guided by the product of adaptation module, *i.e.*, CRISPR RNA (crRNA), may target and degrade invading genetic material (Makarova et al. 2013; Makarova et al. 2011). CRISPR/Cas system has two classes characterized based on the design of effector modules. Class 2 CRISPR/Cas systems have effector complexes with a solitary and big Cas protein originally derived from diverse mobile elements (Makarova et al. 2015). Among CRISPR/Cas systems, protein Cas1 shows the most conservation (Takeuchi et al. 2012) with broad phylogenetic analysis (Makarova et al. 2015; Makarova et al. 2011). Cas2 is trivial and shows less conservation without a consistent phylogeny. Cas1 and Cas2 show co-evolution (Norais et al. 2013; Chylinski et al. 2014). The type VI CRISPR/Cas system Cas1 proteins are dispersed in two clades. The first clade located within type II subtree comprises Cas1 from *Leptotrichia.* The second clade located within the loci of Clostridia involves Cas1 proteins from the locus of C2c2 which belongs to a division of type III-A (Chylinski et al. 2014; Norais et al. 2013). The adaptation module of the Class 2 CRISPR/Cas system has evolved independently from types of Class 1 CRISPR/Cas systems (Shmakov et al. 2015). The evolutionary hierarchy for type VI CRISPR/Cas system is drawn in (Fig. 14.3).

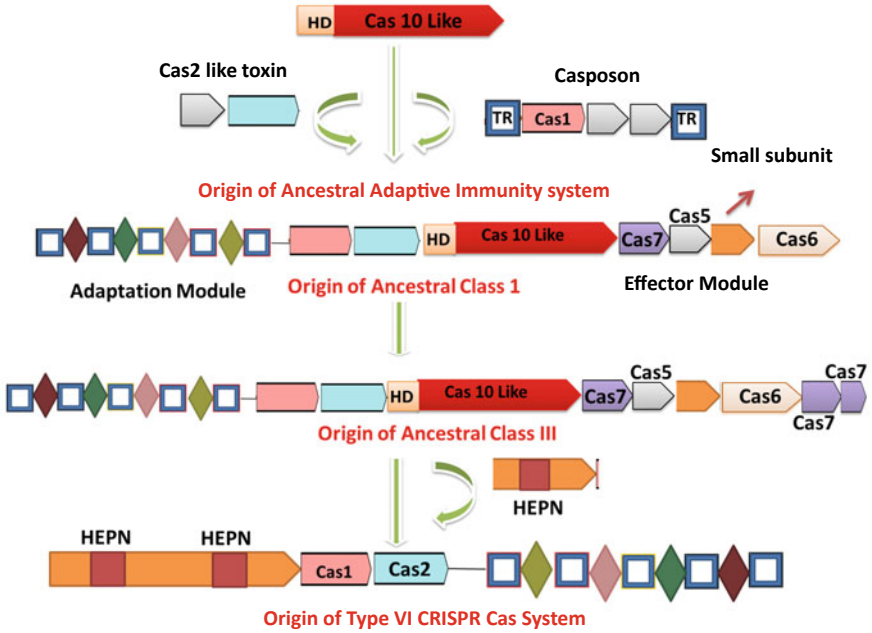

Fig. 14.3 Evolutionary Scenario for Type VI CRISPR/Cas System: Evolutionary scenario of type VI CRISPR-Cas systems initiated with the origin of ancestral adaptive immunity system initiated by fusion of Cas10-like gene with casposon and Cas2-like toxin. Origin of ancestral class I occurred by fission of Cas10-like protein and duplication of RRM domain. Origin of ancestral class III took place with duplication of Cas7. Then annexation and replication of two HEPN domain protein effector modules along with Cas 1 and Cas 2 adaptation modules led to the origin of type VI CRISPR-Cas system. **Abbreviations: TR**: Terminal repeats; **HD**: HD family endonuclease; **HEPN**: Higher Eukaryotes and Prokaryotes Nucleotide-binding domain

14.3.3 Variations of CRISPR/Cas Type VI System (Cas13)

CRISPR/Cas systems have two Classes (1 and 2) which are further categorized into six types (I, II, III, IV, V, and VI) (Makarova et al. 2015; Shmakov et al. 2017). A Class 2 CRISPR/Cas system has II, V, and VI subtypes and integrates both functions of target surveillance and defense into a solitary effector protein (Koonin et al. 2017). Type VI (Cas13) systems have signature solitary effector CRISPR nuclease family comprising gRNA-directed ribonucleases for cleavage of only RNA targets (Yan et al. 2018; Abudayyeh et al. 2016; Smargon et al. 2017; East-Seletsky et al. 2016; Konermann et al. 2018; Shmakov et al. 2015). Cas13 is the signature effector for type VI CRISPR systems. Type VI effectors are among the most deviated CRISPR/Cas proteins with four distinguished subtypes: VI-A (that uses effector Cas13a/C2c2), VI-B (effector Cas13b1/C2c6 and effector Cas13b2), VI-C (effector Cas13c/C2c7), and VI-D (effector Cas13d) (Abudayyeh et al. 2016; Koonin et al. 2017; Shmakov et al. 2015, 2017). But, owing to their great sequence variations, type VI CRISPR/Cas

systems have been subdivided into four subtypes (Shmakov et al. 2015, 2017; Konermann et al. 2018; Smargon et al. 2017; Yan et al. 2018). The signature genes in subtypes VI-A, VI-B, VI-C, and VI-D, encode the effectors Cas13a, Cas13b, Cas13c, and Cas13d, respectively. Therefore, the use of CRISPR repeat sequences as an anchor helped in the identification of Cas13b, Cas13c, and Cas13d (Smargon et al. 2017; Yan et al. 2018; Konermann et al. 2018; Shmakov et al. 2017). Although the type VI effector proteins and the Cas13 subtypes show differences in size and primary sequence, they all share a common feature, which is the presence of two consensus distinct active sites, HEPN domains (Shmakov et al. 2015; Smargon et al. 2017).

The Cas13 effectors adopt a bilobed structure comprising of recognition (REC) and nuclease (NUC) lobes (Liu et al. 2017a) though, the nucleotide base sequence and domain organization intensely diverge from other categories. The REC lobe has a N-terminal domain (NTD) and Helical 1 domain functional for pre-processing and interaction with gRNA (Liu et al. 2017b). Type VI effector modules have two distinctive HEPN ribonuclease domains (with R-X4-H motifs of catalytic residues) in effector module (Smargon et al. 2017; Shmakov et al. 2015, 2017; Konermann et al. 2018; Yan et al. 2018) for cleavage of RNA. The Cas13 variants bear little similarity in nucleotide sequences and are categorized as type VI due to the existence of two terminally located and uniquely spaced conserved HEPN-like domains for every subtype (Shmakov et al. 2015, 2017; Smargon et al. 2017). Domains of HEPN superfamily recurrently occur in ribonucleases of the immune defense system (Anantharaman et al. 2013).

14.3.3.1 Type VI-A (Cas13a/C2c2)

Cas13a (earlier named as C2c2) is a type VI-A ribonuclease which targets and degrades single-stranded phage genome RNA (ssRNA) and does not efficiently cleave dsRNA. It requires only CRISPR RNA to target the ssRNA (Knott et al. 2017). This type of VI CRISPR/Cas system was sequestered from *Leptotrichia shahii* (Severinov et al. 2017). The VI-A locus contains an adaptation module (Cas1, Cas2), two divergent HEPN domains, and CRISPR array (Abudayyeh et al. 2016). The crRNA–Cas13a complex is a bilobed "clenched fist"-like structure with a NUC (nuclease) lobe and crRNA REC (recognition) lobe. The structure and domains of Cas13a vary from other types VI nucleases (Nishimasu et al. 2014; Yamano et al. 2016). NUC lobe of Cas13a contains HEPN (HEPN1 and HEPN2) domains, separated by a linker domain. Helical 3 and Helical 2 domain splits HEPN-1 domains again into two subdomains (Liu et al. 2017b). NUC lobe performs RNase action of Cas13a, pre-processing, and locating gRNA. Other types VI effectors are considerably opposite in nucleotide sequences and structural design of domains (Zhang et al. 2018a, b). Protospacer Flanking Site (PFS) of LshC2c2 comprises A, U, or C nucleotide bases at the 3′ end of the guide sequence (which is 22–28 nt) with complementarity to target nucleotide sequence (Abudayyeh et al. 2016). The base-pair mismatches in the "seed region" decrease C2c2 efficiency (Abudayyeh et al. 2016). LwaC2c2 which is a more active C2c2 ortholog of *Leptotrichia wadei* was

distinguished from LshC2c2 and LwaC2c2 in lacking the 3′ PFS motif and possessing extra C2c2 proteins (Abudayyeh et al. 2017). The LwaC2c2 showed strong cleavage of RNA with 28 bp guide sequence and there was no cleavage activity with guide sequences of less than 20 bp. LwaC2c2 has been shown to cleave mammalian cell transcripts by targeting *KRAS, CXCR4, PPIB* genes, and *Gaussia luciferase* reporting genes (Abudayyeh et al. 2017) and shown to target Turnip mosaic virus (TuMV) in tobacco (*Nicotiana benthamiana*) (Aman et al. 2018a). Using LwaCas13a, more than 50% knockdown was observed in genes like *EPSPS, HCT*, and *PDS* in protoplasts of *Oryza sativa* (Abudayyeh et al. 2017). The mutated dLshC2c2 and dLwaC2c2 versions of LshC2c2 and LwaC2c2 down-regulate gene expression (Abudayyeh et al. 2016, 2017). The crystal structures of proteins of Cas13a available in public domains are LshCas13a (Cas13a of *Leptotrichia shahii*), LbaCas13a (Cas13a of *Lachnospiraceae bacterium*), and LbuCas13a (Cas13a of *Leptotrichia buccalis*) (Liu et al. 2017a, b; Knott et al. 2017).

14.3.3.2 Type VI-B (Cas13b/C2c6)

Cas13b is an RNA-guided and RNA-targeting effector enzyme. Cas13b was discovered by use of computational approaches in gram-negative bacterial species of *Porphyromonas* sp. and *Prevotella* sp. (Smargon et al. 2017) and owing to parallel nature to Cas13a was named as Cas13b (previously named as C2c6) and apportioned to Type VI subtype VI-B (Smargon et al. 2017). The Cas13b and Csx27 were sequestered *in vivo* from *Bergeyella zoohelcum* and subjected to functional characterization in *E. coli* (Smargon et al. 2017). Type VI-B CRISPR/Cas system is devoid of universal Cas1 and Cas2 proteins but encompasses two formerly uncharacterized associated proteins, namely, Csx27 and Csx28, phylogenetically related to Cas13b but lack sequence resemblance with Cas13a effector nuclease (Smargon et al. 2017). Based on the existence of these two supplementary accessory proteins (Csx27 and Csx28), the subtype VI-B is subdivided into VI-B1 and VI-B2 categories (Smargon et al. 2017; O'Connell 2019). The binding of Cas13b with proteins Csx27 and Csx28, represses and enhances the Cas13b-mediated RNA target cleavage activity, respectively (Smargon et al. 2017; O'Connell 2019). The Cas13b protein has two HEPN domains that are positioned at N and C protein terminals (Shmakov et al. 2017). The Lid domain in Cas13b covers the 3′ end of gRNA with two charged beta-hairpins which give stability to proteins (Slaymaker et al. 2019). The Cas13b causes processing of CRISPR RNA and needs paired-sided protospacer flanking sites and the secondary structure of RNA to target RNA (Smargon et al. 2017). Cas13b endonuclease assumes an open conformation that permits target RNA to advance into the central accessible passage of endonuclease for target RNA (Slaymaker et al. 2019). But Cas13a and Cas13d vary and include a solvent-exposed fissure for locking RNA target (Slaymaker et al. 2019). Investigation of the RNA-targeting property in eukaryotes discovered Cas13b ortholog from *Prevotella* sp. with constantly greater efficiencies than LwaCas13a (Cox et al. 2017). PspCas13b also lacked collateral

damage of RNA in eukaryotes, lacked the need for PFS, and showed similar specificity and amenability to multiplexing like Cas13a. Because of these characteristics, PspCas13b is currently the first choice for targeted RNA cleavage (Cox et al. 2017). The Cas13b and CsX27 have a predilection for 5′ PFS of A, U, or G and 3′ PFS of NAN or NNA. The modified Cas13b version, dCas13b, is fused with ADAR2 deaminase domain (ADARDD) and led to A– I replacement when introduced into mammalian cells. This has applications in the treatment of human disorders and protein modification for validating the genetic functions across different organisms, together with plants (Cox et al. 2017).

14.3.3.3 Type VI-C (Cas13c/C2c7)

It was identified using a computational approach in *Fusobacteria* and *Clostridi*. The average size of VI-C is 1120 amino acids. The adaptation module is devoid of Cas1 and Cas2 proteins. There is not much research work available on this type of Cas13.

14.3.3.4 Type VI-D (Cas13d)

Using a computational pipeline for genome and metagenome sequences, a novel Cas13 subtype designated as Cas13d—a type VI-D CRISPR/Cas effector was identified (Konermann et al. 2018). Cas13d endonucleases are the smallest with an average size of 930 aa in human cells, 20–30% smaller compared to other Cas13 subtypes, enabling bendable packing into size-constrained medical viral vectors, for example, adeno-associated virus (AAV) (Konermann et al. 2018; Yan et al. 2018). The average dimension of Cas13d protein is 190–300 amino acids (Konermann et al. 2018). Cas13d HEPN-2 domain has two R-X4-H HEPN motifs for degradation of target RNA, catalytic site for pre-crRNA processing, however, owns slight global comparison to amino acid sequences of Cas13a and Cas13b (Konermann et al. 2018; Smargon et al. 2017; Yan et al. 2018). Some 77% of *Cas13d* genes are located nearby CRISPR arrays and in 19% of them, *Cas1* and *Cas2* genes are located in surrounding locales. The CRISPR arrays nearby *Cas13d* genes comprises 198 spacers out of which 182 are unique (Konermann et al. 2018). UrCas13d which is a type VI CRISPR effector structure is compressed wherein the REC and NUC lobes appear slightly blurred. REC lobe has NTD and Helical-1 domain while NUC lobe has domains of HEPN-1 and HEPN-2, and Helical-2. The HEPN-1 comprises 10 α-helices whereas HEPN-2 domain contains 11 α-helices. These two HEPN domains show interaction by helix-α3 and helix-α28. Helical-1 and Helical-2 domains have eight α helices each which bind around HEPN-2 helix-α28 (Yan et al. 2018). The Cas13d is an RNA-guided ribonuclease which has a compact REC lobe contrary to Cas13a, causing the 3′ region of gRNA to bulge out of the protein and uncovered for solvent (Zhang et al. 2018b). WYL1 is an accessory protein, that has been identified in type VI-D systems (Yan et al. 2018) with a possible function of enhancing

cleavage of Cas13d by linking target RNA with effector module (Zhang et al. 2019a). WYL1 possesses helix-turn-helix and WYL domains in contrast to Csx27 and Csx28 with transmembrane domains (Smargon et al. 2017; Yan et al. 2018). Zhang et al. (2018b) reported the structure of EsCas13d (*Eubacterium siraeum*) in crRNA-bound and target-bound positions. RspCas13d (*Eubacterium siraeum*) and EsCas13d (*Ruminococcus* sp.), which are Cas13d orthologs, show activity in processing of RNA, target RNA cleavage and collateral damage, and lack in target-flanking sequences. Cas13d displayed vigorous activity in the degradation of RNA targets (Cox et al. 2017) and binding in human cells (Konermann et al. 2018). However, VI-A, VI-C CRISPR/Cas systems show variable dependence on PFS for ssRNA targeting and show auto-processing of pre-crRNAs into mature CRISPR RNA-enzyme complex (Abudayyeh et al. 2016, 2017; Cox et al. 2017; East-Seletsky et al. 2016, 2017; Gootenberg et al. 2017; Smargon et al. 2017). The Cas13d pre-crRNA site/domain of processing is identified and depend on divalent Mg^{2+} cations interaction with nucleotides toward 3′ crRNA repeat region to generate mature crRNA (East-Seletsky et al. 2016; Zhang et al. 2018b; Knott et al. 2017). The variations in structural architecture of Type VI CRISPR/Cas systems are given in (Fig. 14.4).

14.3.4 Mechanism of Type VI CRISPR/Cas System

The general mechanism of CRISPR has four distinctive phases: adaptation (spacer acquisition), pre-crRNA expression, maturation (biogenesis of crRNA), and the interference (RNA targeting). The progression of adaptation and expression is nearly analogous for various CRISPR categories, but the biogenesis process of crRNA, target type, and mechanism of targeting show a divergence in diverse types of CRISPR/Cas systems.

The four phases of type VI CRISPR/Cas system are discussed below.

14.3.4.1 Adaptation Phase/Spacer Acquisition in Type VI CRISPR/Cas System

Adaptation is the initial stage in the process of weapon designing against foreign invading RNA bacteriophages. In the adaptation phase, Cas1/Cas2 complex with two dimers of Cas1 and solitary dimer of Cas2, picks and processes a region of invading viral (RNA bacteriophages) for spacer (protospacer) generation and directionally integrates the generated protospacer as a novel spacer at the position of leader-first repeat junction in CRISPR array which is separated by repeat sequences and pre-existing spacers, consequently generating a memory of annexed genetic material (Fig. 14.5).

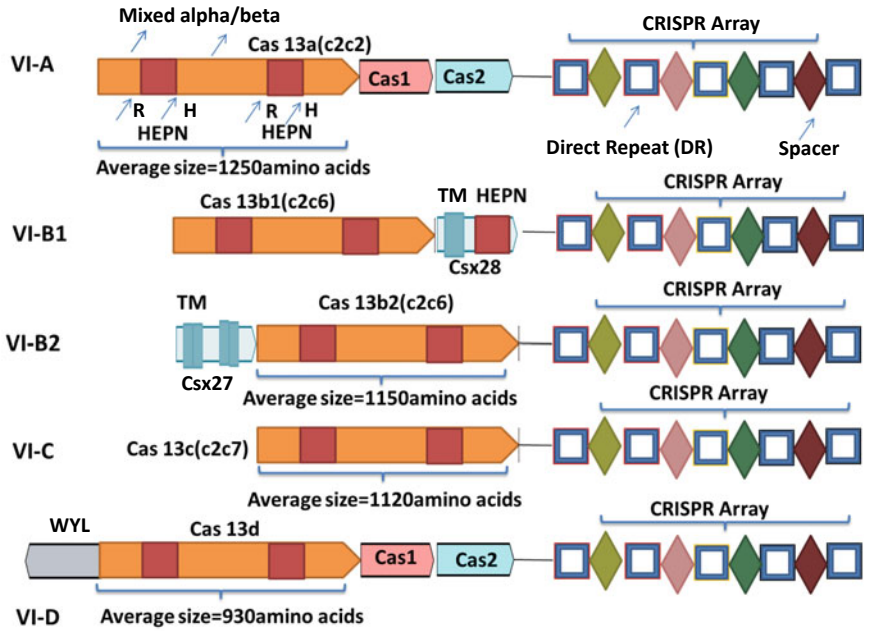

Fig. 14.4 Structural Variations of Type VI CRISPR/Cas systems: VI-A *Leptotrichia shahii;* VI-B1: *Prevotella buccae*; VI-B2: *Bergeyella zoohelcum*; VI-C: *Fusobacterium perfoetens*; VI-D: *Ruminococcus bicirculans*. The principal organization domains of CRISPR-Cas locus are adaptation module/an operon of Cas genes and CRISPR array of short repeats interspersed with spacers. Domain organization involves Cas genes which are represented by arrows and labelled with gene names along with Cas1 and Cas2 proteins in VI-A and VI-D whereas VI-B2 and VI-B1 have Csx27 and Csx28, respectively. Csx27 genes are not always found within the VI subtype. Two HEPN domains with conserved residues are present in VI-A. The average size of Cas13 protein subtypes is indicated. The size for Cas13B includes both VI-B1 and VI-B2 subtypes. Within each CRISPR array squares represent DR, while diamonds represent spacer sequences which are derivatives of acquired genomic sequences of invading bacteriophage. **Abbreviations: HEPN**: Higher Eukaryotes and Prokaryotes Nucleotide-binding domain; **WYL**: WYL domain; **TM**: predicted transmembrane-spanning region; **DR**: Direct Repeats

The course of adaptation may be further subdivided into two stages: (i) the scan and seizure of a sequence known as protospacer from invading DNA and (ii) the integration of captured protospacer into bacterial CRISPR array as the newest spacer (McGinn and Marraffini 2019; Amitai and Sorek 2016). The first step is directed by Cas1–Cas2 complex (Wang et al. 2015; Nunez et al. 2015a) and facilitated by Rec BCD (Levy et al. 2015; Ivancic-Bace et al. 2015). The various types of CRISPR/Cas systems choose protospacers preferentially (Wang et al. 2015), involving Cas3 and Cas4 as well (Shiimori et al. 2018; Kieper et al. 2018; Lee et al. 2018; Kunne et al. 2016). According to Koonin et al. (2017) the Cas1 and Cas2 show conservation in various CRISPR/Cas systems. The bioinformatics analyzes (PSI-BLAST and HH pred) identified that subtype VI-A loci of type VI system has adaptation

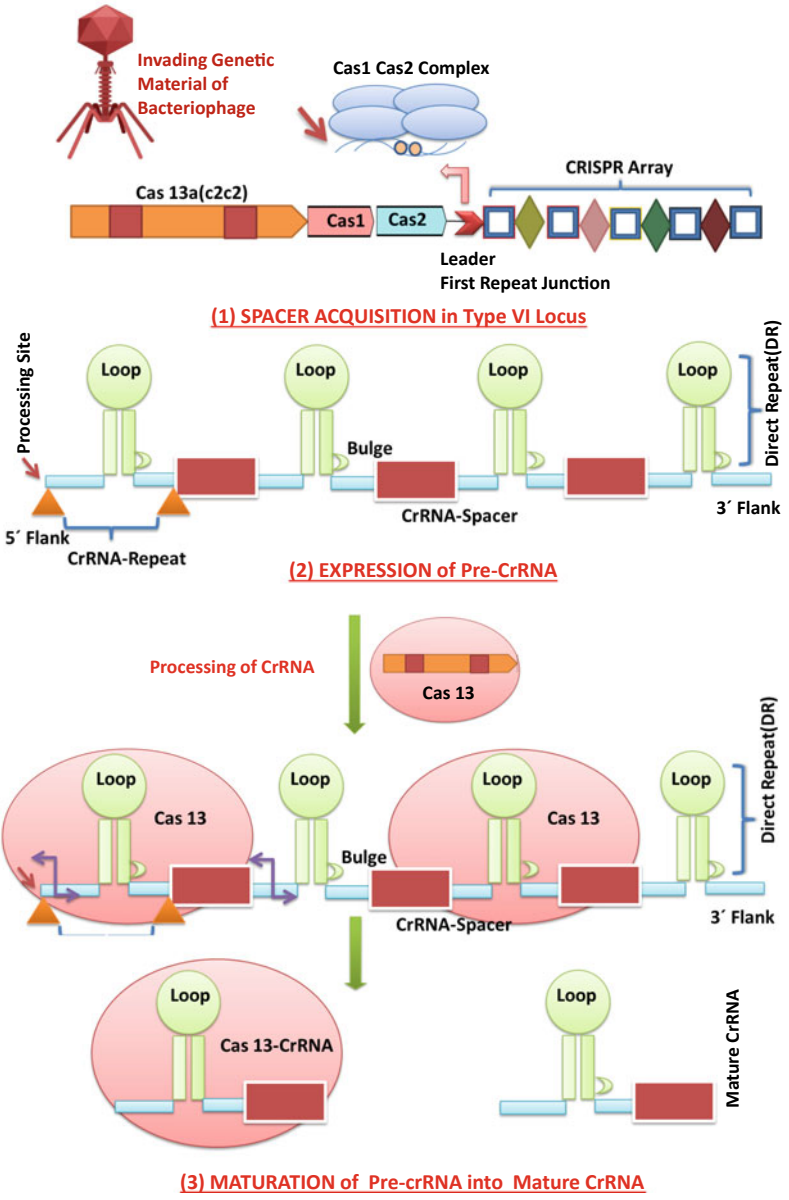

Fig. 14.5 Mechanism of Type VI CRISPR-Cas Systems: Type VI CRISPR/Cas functions in four stages: (1) Adaptation or spacer acquisition in type VI locus wherein short fragments of invading RNA are acquired by Cas1–Cas2 and integrated at leader sequence as new spacers by adaptation. (2) Expression or transcription of pre-crRNA. (3) Processing involves maturation of pre-crRNA into mature crRNA by Cas13 effector proteins. Mature crRNAs and Cas proteins assemble into crRNP surveillance complex. (4) Interference of invading RNA takes place by its sequence complementarity with crRNA guide protospacer through cis target cleavage and non-specific promiscuous trans target cleavage causing programmed cell death or dormancy induction

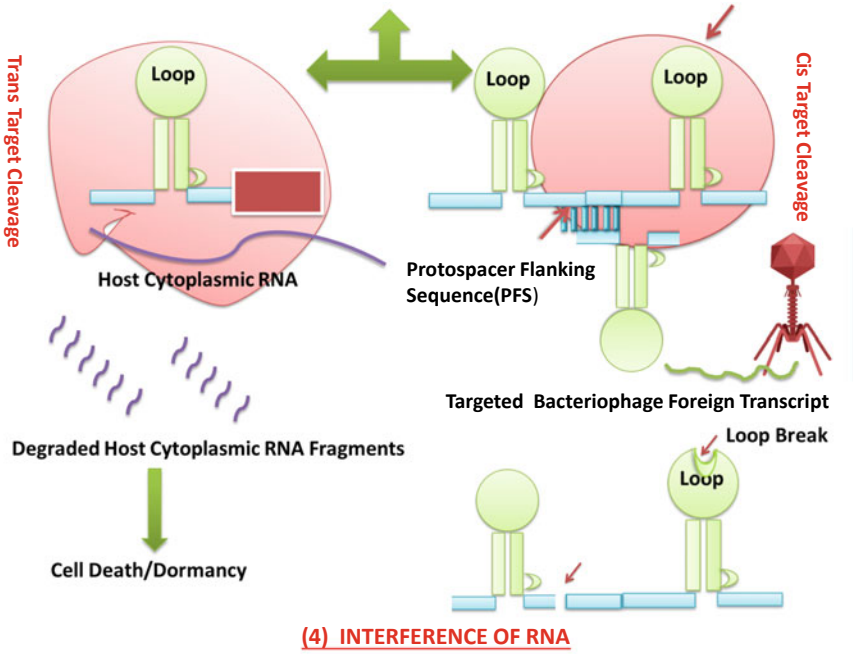

Fig. 14.5 (continued)

modules with which these directly capture new protospacers from RNA bacteriophages (Shmakov et al. 2015). But that acquisition course from bacteriophage RNA necessitates encoding reverse transcriptase enzyme (Toro et al. 2017; Silas et al. 2016). *Lachnospiraceae bacterium* MA 2020 is the only exception in type VI CRISPR/Cas systems that encodes reverse transcriptase (Shmakov et al. 2015). An alternative option suggests that the type VI CRISPR/Cas system depends on the adaptation module and CRISPR arrays of additional categories of CRISPR/Cas systems of bacteria (Silas et al. 2017). But, the precise spacer acquisition mechanism in type VI CRISPR/Cas system is still unexplored. The conserved adaptation module of CRISPR which includes Cas1 and Cas2 proteins in LshC2c2 loci helps in spacer acquisition. The C2c2 is devoid of reverse transcriptases, which facilitate the acquisition of the protospacer in the type III CRISPR/Cas system (Silas et al. 2016), so some supplementary host (bacterial) or viral factors might be assisting in RNA spacer acquisition. Type VI CRISPR/Cas systems have DNA spacer acquisition analogous to other categories of CRISPR/Cas. However, they target their respective RNA transcripts, causing programmed cell death and abortive infection. Then, the novel spacer is processed and gets subjected to insertion in the locus nearby leader sequence of CRISPR array by the help of Cas1–Cas2 complex (Nunez et al. 2014; Wright et al. 2017; Xiao et al. 2017; Nunez et al. 2015b) which helps the bacteria to develop immunity against the new invaders (Modell et al. 2017; McGinn and Marraffini 2016; Weinberger et al. 2012).

14.3.4.2 Expression Phase/Expression of Pre-CRISPR RNA in Type VI CRISPR/Cas System

In the expression phase, the CRISPR locus undergoes transcription by a promoter located upstream of the AT-rich leader sequence and forms a lengthy transcript of pre-crRNA. The CRISPR locus does not transcribe tracrRNA complementary to repeat sequences in transcripts of the crRNA.

14.3.4.3 Maturation Phase/Maturation of the CRISPR RNA in Type VI CRISPR/Cas System

In the type VI CRISPR/Cas system, the maturation of pre-crRNA into mature crRNA (for RNA recognition) which has a transcribed spacer sequence linked to partial repeat sequence, is executed by Cas13 effector protein itself, instead of a pre-crRNA processing nuclease in a metal independent mode (except in VI-D) (East-Seletsky et al. 2017; Liu et al. 2017b; Shmakov et al. 2015; East-Seletsky et al. 2016). The Cas13 effector protein degrades the pre-crRNA at permanent locations upstream of the stem–loop structure (shaped due to palindromic repeated sequences) in tracrRNA in an independent manner or devoid of other host factors (Shmakov et al. 2015). Pre-crRNA repeated sequences form a bulged stem-looped structure. The bulge is conserved in nature and indispensable part of mature crRNAs in Cas13a effector proteins. The interference of the bulge hampers the degradation of the target RNA, however, processing of pre-crRNA is not affected (Liu et al. 2017a; Knott et al. 2017; Liu et al. 2017b). According to Liu et al. (2017a), the bulge, stem, or loop alterations in the handle may influence cleavage of target by Cas13 endonuclease. The nonconservation of some of the residues and cleavage sites leads to the varying length of the 5′ handle among diverse Cas13 homologs (Liu et al. 2017b; East-Seletsky et al. 2016). The subtypes VI-A, VI-C, and VI-D of type VI contain mature crRNAs containing a repeat-sequenced conserved handle forming a stem–loop on their 5′ end and a spacer which shows flexible length (East-Seletsky et al. 2016; Shmakov et al. 2015; Liu et al. 2017b). Quite the reverse, subtype VI-B of type VI CRISPR/Cas system matures crRNAs having the handle on the 3′ end (Smargon et al. 2017). Thus the mature CRISPR RNA in types VI-A, VI-C, and VI-D have 5′ handle and the mature CRISPR RNA in VI-B owns 3′ handle (Konermann et al. 2018; East-Seletsky et al. 2016; Cox et al. 2017; Liu et al. 2017b). The spacer <20 nt obliterated the Cas13cleavage activity without having any effect on its capability of binding of RNA (East-Seletsky et al. 2016; Liu et al. 2017a, b). The maturation of crRNA is not indispensable for the activity of type VI CRISPR/Cas system as unprocessed pre-crRNA is also capable of RNA target recognition (East-Seletsky et al. 2017). The mature crRNAs form functional ribonucleoprotein (RNP) complexes with Cas protein(s). The gRNA (crRNA) of type VI CRISPR/Cas system has a stem–loop containing direct repeat sequences lined by spacer region. Digits of Watson–Crick base-pairing inside stem–loop, the sequence length of the direct repeats and comparative location of direct repeats and spacers differ in VI-A, VI-B, VI-C, and

VI-D (O'Connell 2019). Some type VI-B loci encode additional functional crRNA which possesses a direct repeat zone of >80 base nucleotides (Smargon et al. 2017). Type VI-A, VI-D, and perhaps VI-C accept bulge at the base of the crRNA stem (Liu et al. 2017b; Zhang et al. 2018b) which dictates the proper dual RNase activity. The stem–loops of type VI-D have numerous base-pairings following non-Watson–Crick rule (Zhang et al. 2018b). The NTD, Helical-1 domain, and HEPN2 domain constitute a constricted cleft with a positive charge which fixes the 5′ repeat sequence of the end portion of the bound crRNA (5′ handle), while 3′ end of crRNA is enclosed by the domain of Helical-2. NUC lobe encloses the first rare guide nucleotides of the crRNA, while the central zone of crRNA remains solvent-exposed. The pre-crRNA processing and maturation into mature crRNA are followed by the anchorage of its 5′ and 3′ ends inside the complex for binding of target RNA. Helical-1 domain, when subjected to mutagenesis at positively charged residues like Arg438 plus Lys441 in LshCas13a, abolished the processing of pre-crRNA. This signifies the role of the Helical-1 domain in the development and binding of 5′—handle of the crRNA (Liu et al. 2017b; Abudayyeh et al. 2016). The diagram emphasizes the chief parallels and alterations in four different types VI crRNAs (Fig. 14.6).

14.3.4.4 Interference Phase/Bacteriophage RNA Interference in Type VI CRISPR/Cas System

In the interference phase, the crRNA–Cas RNP hybrid complex scans transcripts for complementary repeat region sequences of invading RNA target by complementary base-pairing and the crRNA makes base pairs with protospacer of invading RNA target. The RNA interference in the type VI system is guided by solitary CRISPR RNA. According to Shmakov et al. (2015) the respective effector proteins are designated as Cas13a, Cas13b, Cas13c, Cas13d in VI-A, VI-B, VI-C, and VI-D. Type VI effector complex Cas13 possesses only one protein with functional HEPN domain positioned toward terminal ends of Cas13; one with pre-crRNA processing activity and another with nucleolytic cleavage activity against RNA target/RNase activity, which exclusively degrades the bona fide target substrate, i.e., RNA (Shmakov et al. 2017; Koonin et al. 2017; Shmakov et al. 2015; Abudayyeh et al. 2016; Konermann et al. 2018; Smargon et al. 2017; Tamulaitis et al. 2017). RNase activity of Cas13a for processing of crRNA and cleavage of the target was revealed by various *in vitro* assays and structural investigations (Liu et al. 2017a, b). According to Smargon et al. (2017) and Abudayyeh et al. (2016), a heterologously expressed type VI in *E. coli* provides immunity against ssRNA phage (MS2), which lacks DNA stage in the lifecycle, signifying that ssRNAs is the exact target of Cas13 but, it was complemented with a growth suppression phenotype. Effector protein Cas13 and cRNA complex exhibits nucleolytic activity only after binding to the targeted ssRNA. Remarkable modification in the conformation of the Cas13a occurs after RNP complex (crRNA-targeted A-form dsRNA duplex) formation takes place, through the binding of crRNA with targeted RNA for recognition of target ssRNA (Liu et al. 2017a; Zhang et al. 2018a; Liu et al. 2017b). The conformational change helps in accommodating the proliferating duplex

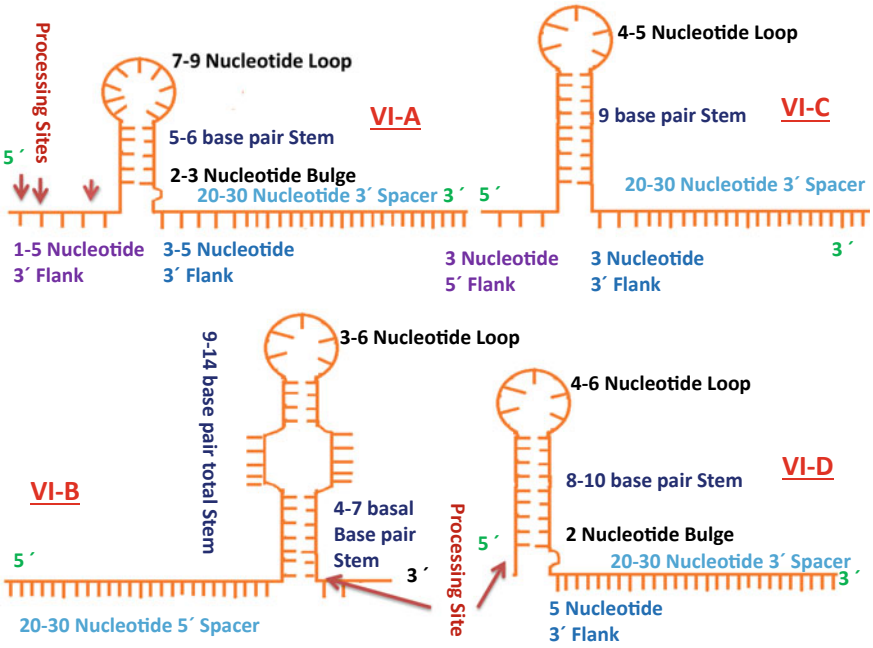

Fig. 14.6 Diverse Emphasized Characteristics of crRNAs in four variants of Type VI: Schematic representation of main parallels and variance in features of VI-A, VI-B, VI-C, VI-D crRNAs. Spacer length varies in VI-A, VI-C, VI-D from 20 to 30 nt 3′ whereas VI-B has 20–30 nt 5′ spacer on expression in *E. coli*. Stem and nucleotide loop length also varies. Stems in VI-A and VI-D may possess non-Watson–Crick base-pairing, mismatches, or bulges. VI-B has a big noncomplementary area in the mid stem. Different VI-A pre-crRNA processing sites are indicated by red arrows

inside a channel of the NUC lobe with a positive charge. The catalytic residues of two HEPN domains move in adjacent proximity to each other and thereby generate a solitary composite catalytic site of RNA cleavage (Liu et al. 2017a). This catalytic site is formed at a particular distance to RNP complex on the exterior of the protein, so not only the targeted RNA is cleaved, but, correspondingly some additional ssRNA and bacterial cells own RNA which is present nearby RNP complex, also get degraded. So, this extremely accessible active site not only degrades long RNA target in the configuration of *cis* but also confers uninhibited RNase activity to non-target RNAs in *trans*, which is known as collateral damage/cleavage (Abudayyeh et al. 2016; Yan et al. 2018; Liu et al. 2017a; Smargon et al. 2017). The non-specific degradation of RNA is conferred by the HEPN domain in Cas13. The collateral damage helps to cope with bacteriophage infection and abortive infection by cleaving all foreign cell RNA and in turn blocks phage replication to protect neighboring cells (Makarova et al. 2012).

The nucleolytic degradation of RNA target via Cas13 variants is controlled and modulated by the accessory proteins. RNA target cleavages by Cas13 RNase activity

are repressed by prolonged complementarity between the RNA target and the handle which flanks spacer (Meeske and Marraffini 2018). In type VI-B systems, specific modulation by Csx27 gene repressor protein regulates repression of the RNase action of the Cas13b HEPN domain, whereas the *Csx28* gene stimulator protein regulates enhancement in the RNase activity of Cas13b HEPN domain (Smargon et al. 2017). Yan et al. (2018) opined that the RNase action of Cas13d improves through an ortholog of WYL1. RNase activity of Cas13, for the maturation of crRNA and Cas13 RNase activity for target RNA cleavage are independent of each other. When catalytic residues of HEPN domains were subjected to mutations, catalytically inactive alternate of Cas13, *i.e.*, dCas13, was generated but the abilities of crRNA maturation (Cox et al. 2017; Liu et al. 2017b) and binding to target RNA remained intact (Konermann et al. 2018). When locales exterior to HEPN domain like R1079A/K1080A of *Leptotrichia buccalis* Cas13a were subjected to mutations, the ability to process mature crRNAs got obliterated but the target degradation remained unaffected (East-Seletsky et al. 2016, 2017). crRNA-directed degradation of RNA takes place via Cas13 effector protein at cognate protospacer which is matching to the spacer of the crRNA. Cleavage of any specific RNA target by various crRNAs generates identical patterns (Smargon et al. 2017; Abudayyeh et al. 2016; Yan et al. 2018). Subfamily Cas13 RNAse activity shows specific variance in preference to a nucleotide, for example, adenosine or uridine-rich RNAs get targeted (Abudayyeh et al. 2016; East-Seletsky et al. 2016, 2017). Cleaving of the target by Cas13a RNA-guided RNase is both sequence and structure-dependent and cleaves whichever ssRNA by identifying a 28 nt region on crRNA, however, cleavage is not site-directed. The Cas13a cleaves RNA by conserved residues present in two HEPN domains, contrary to the mechanism of catalysis in other recognized RNases (Tamulaitis et al. 2014; Benda et al. 2014). Type VI CRISPR/Cas systems apply a safety-lock mechanism for inhibiting system activation by the host bacteria's RNA.

The CRISPR/Cas systems are PAM-dependent and PAM-independent based on the ssRNA target predilection of PAM/rPAM/PFS. In Cas13a and Cas13b, PFS predilection in ssRNA is indispensable for cleavage of RNA; however, Cas13d is PAM-independent. The PAM-dependent Cas13a needs H at 3′ PFS, whereas PAM-dependent Cas13b homologs necessitate NAN or NNA at 3′ PFS as well as D at 5′ PFS (both 3′ and 5′ PFS) while Cas13c and Cas13d have none (Abudayyeh et al. 2016, 2017; Zhang et al. 2018a; Smargon et al. 2017). The non-GPFS motif 3′ positioned at 3′ end of the protospacer is required for robust RNAi in LshCas13a (Abudayyeh et al. 2016).

Semenova and co-workers (2011) reported that the seed region is a conserved location in the spacer proximal to PAM. The mismatches external to seed zone are permitted by Cas13 effectors to variable grades. Type VI CRISPR/Cas system possesses the spacer typically about 30 nt long whereas the binding and cleavage seed zone of RNA fluctuates. According to Liu et al. (2017a) and Tambe et al. (2018), the central seed region for binding of RNA in Cas13a CRISPR/Cas system extends from 5 to 8 nt whereas Cox et al. (2017) reported that the degradation seed zone extends from 13 to 24 nt. Cox et al. (2017) believed that in Cas13b CRISPR/Cas system seed locale of degradation of RNA extends from 12 to 26 nt whereas RNA binding seed

zone nucleotide range is indistinct. The seed zone with conservation is not required for Cas13d-mediated degradation of ssRNA (Zhang et al. 2018b). When the target region matching the central seed region of crRNA was subjected to mutagenesis with two mismatches, the activation of Cas13 both *in vitro* and in vivo got averted. The investigation of the crystal structure of target-bound LbuCas13a supported it (Liu et al. 2017a; Abudayyeh et al. 2016). To conclude, RNA viruses epitomize only a negligible portion of the prokaryotic virome (Koonin et al. 2015). Therefore, the type VI CRISPR/Cas system might predominantly stimulate toxic action in reaction to RNA transcripts transcribed by invader DNA (Sheppard et al. 2016; Niewoehner and Jinek 2016). The detailed mechanism of type VI CRISPR/Cas Systems is depicted diagrammatically in (Fig. 14.6).

14.3.5 Potential Applications of CRISPR/Cas13

14.3.5.1 RNA Targeting

Plant viruses are important biotic factors that infect a wide variety of plant species, cause several diseases, resulting in huge losses of yield in terms of quality and quantity (Nicaise 2014). Researchers adopted several strategies such as engineering plants with antiviral genes, silencing of virus genetic material through RNAi technology to develop virus resistance crop plants (Baulcombe 1996; Simon-Mateo and Garcia 2011; Younis et al. 2014). However, transgenic plants developed with these strategies have several limitations (Prado et al. 2014). Recent studies in bacterial cells, humans, and plants suggest that CRISPR/Cas13a can be programmed to target-specific RNA molecules (East-Seletsky et al. 2016; Abudayyeh et al. 2017). In plants, initially, Cas13a of bacteria *Leptotrichia shahii* was used to target three rice (*Oryza sativa*) genes, *i.e.*, 5-*enolpyruvylshikimate-3-phosphate synthase (EPSP)*, a lignin biosynthetic gene *hydroxycinnamoyl-CoA shikimate/quinate hydroxycinnamoyl transferase (HCT*), and a *phytoene desaturase* (*PDS*) gene. A plant codon-optimized LwaCas13a was cloned into a plant transformation vector and three guides for each target transcript were cloned into a guide vector and both the vectors were co-transfected into rice protoplasts. Transfected protoplasts were measured for target transcripts and found that seven out of the nine guides knocked down more than 50% of the transcripts and guide one of the *PDS* transcript exhibited a maximum of 78% target transcript knockdown, which is comparable with the RNAi mechanism (Abudayyeh et al. 2017). Thus, this data indicates that Cas13a protein can be reprogrammed with guides to arbitrate nuclear targeted multiplexed knockdown in plant cells (Abudayyeh et al. 2017).

To further test, whether the expression of Cas13a protein target viral RNA of plants and provides virus resistance to plants, two individual experiments were conducted by transforming a plant codon-optimized *Leptotrichia shahii* (LshpCas13a) into *N. benthamiana* leaves for transient and transgenic expression. Four guides such as green fluorescent protein (GFP)1, GFP2, the helper component proteinase silencing

suppressor (HC-Pro), and coat protein (CP) sequences were used to target GFP-tagged Turnip mosaic virus genome (TuMV-GFP). The TuMV-GFP transcripts levels were measured in transient and transgenics plants by observing the GFP signal in the leaves under UV light after seven days of post infiltration. The data clearly shows that in both of the experiments ~50% reduction in the transcript levels was observed for HC-Pro and GFP2 guides and low but detectable levels of reduction in transcript signal for CP and GFP1 (Aman et al. 2018a). This experiment provides evidence that CRISPR/Cas13a proteins can be engineered for stable expression in plants and Cas13a protein significantly interferes with the plant virus RNA genomes, thereby providing resistance to plants against the viruses (Aman et al. 2018a; Mahas and Mahfouz 2018). In another similar study, stable integration and expression of LshpCas13a protein and crRNAs, corresponding to different regions of TuMV-GFP virus, into *Arabidopsis thaliana*, provided heritable immunity against TuMV virus up to the T2 generation. This data suggest that CRISPR/Cas13 strategy would be a powerful antiviral strategy to tackle plant viruses (Aman et al. 2018b).

Besides LshpCas13a, several well-characterized variants of Cas13 proteins, such as LwaCas13a (*Leptotrichia wadei*), BzCas13b (*Bergeyella zoohelcum*); PspCas13b (Prevotella sp. P5-125), and CasRx13d (Ruminococcus flavefaciensXPD3002) were tested to identify better variants against the plant viruses (Mahas et al. 2019). Transient assays were conducted in tobacco plants, using the tobacco mosaic virus (TMV)-RNA-based overexpression (TRBO-G) system. The virus RNA interference data suggest that when compared with LshpCas13, all the new variants (LwaCas13a, PspCas13b, and CasRx13d) are more efficient and CasRx emerged as a potential candidate against plant virus (Mahas et al. 2019).

14.3.5.2 RNA Tracking

RNA molecules, very often, can be visualized through the techniques "single-molecule fluorescence in situ hybridization (smFISH)," however; this technique cannot visualize RNA in living cells, as it is very difficult to remove unbound probes (Femino et al. 2003; Raj et al. 2008; Yang et al. 2019). Several research groups have come up with some alternative technologies such as stem–loop labeling and fluorescence tagging with MS2-MCP system (Ben-Ari et al. 2010; Larson et al. 2011; Wu et al. 2012); fluorogenic RNA aptamers (Paige et al. 2011; Filonov et al. 2014); and use of molecular beacons, fluorogenic oligonucleotide probes (Chen et al. 2017; Tyagi and Kramer 1996). However, all these techniques are expensive and need several transcripts. Modern techniques such as engineered RNA-targeting Cas9 (RCas9) were used to detect housekeeping mRNAs (i.e., β-actin) which are available in greater quantity in the cells (Nelles et al. 2016; Batra et al. 2017). However, this method requires technical expertise and so far there are no reports mentioning the application of this method for other types of RNAs (Yang et al. 2019). The CRISPR/Cas13 proteins can be used as alternative tools for tracking of RNAs as they specifically bind to RNA molecules. A biotin-labeled deactivated variant of Cas13a protein dLwaCas13a was successfully used to detect highly

expressed *β-actin* transcript using a negative feedback (NF) system (Abudayyeh et al. 2017). Besides the high abundant transcripts, moderately or low abundant transcripts can also be detected through deactivated Cas13b protein. The enhanced-GFP (eGFP)-tagged deactivated Cas13b proteins (eGFP, dPspCas13b, and dPguCas13b) were used to track, less abundant transcripts such as *NEAT1*, *MUC4*, *GCN4*, and *SatIII* transcripts (Yang et al. 2019; Davis and O'Connell 2020). The LwCas13a was combined with isothermal amplification and developed an attomolar sensitivity for tracking single-base mismatch termed as Specific High-Sensitivity Enzymatic Reporter UnLOCKing (SHERLOCK) (East-Seletsky et al. 2016; Abudayyeh et al. 2016; Gootenberg et al. 2017). The SHERLOCK technology was successfully applied to detect the glyphosate resistance gene, CP4 EPSPS (*Agrobacterium* sp. strain CP45-enolpyruvylshikimate 3-phosphate synthase) and a housekeeping gene LE4 (Lectin) in the crude soybean extracts (Abudayyeh et al. 2019a). This portable detection system would allow to detect the pests and pathogens at the early stage (Abudayyeh et al. 2019b).

14.3.5.3 RNA Editing

The engineered Cas13 system was successfully applied to edit programmable A to I system (A to I replacement version (REPAIR) in humans. The deactivated Cas13b protein was fused with a mutation-enhanced deaminase domain of ADR1 and ADR2 of humans to develop engineered REPAIRv1 and REPAIRv2. The data generated indicates that REPAIRv1 has a high editing capacity than REPAIRv2 (Cox et al. 2017). Recently, a synthetic adenine deaminase domain of ADAR2 (ADAR2dd) was fused with dCas13 and developed as a programmable RNA Editing for Specific C to U Exchange (RESCUE) (Abudayyeh et al. 2019b). However, since it is a new system, further study is needed in plants to know the efficacy of Cas13 proteins as RNA editing molecules.

14.4 Potential Limitations of CRISPR/Cas13

Even though the CRISPR/Cas13 has few biotechnological and agriculture applications as discussed in the previous section, there are some limitations in the usage of the technology. Though it was not observed in human cells and plant studies, but in some *in vitro* studies and bacterial cells, it was observed that the active Cas13 also turns on its collateral RNase activity, followed by binding to a target transcript, resulting in the degradation of non-target RNAs also (East-Seletsky et al. 2016). Similar to Cas9, Cas13 has some off-target effects (Wang et al. 2019). Another important concern about Cas13 is, by modifying the spacer in crRNA, cleavage sites and cleavage pattern of particular target transcript cannot be changed, it is fixed for a particular target ssRNA (Abudayyeh et al. 2016; Smargon et al. 2017; Konermann et al. 2018). RNA targeting, using dCas13 tagged with an epitope, is an important

application of Cas13. The major concern of this application is affinity and specificity of the RNA binding domain of Cas13 (Wang et al. 2016). As there are no specific guidelines yet for designing gRNAs for target RNA tracking using dCas13, the only need is to depend on the structure of target RNA while designing the gRNAs (Yang et al. 2019). Another apprehension about Cas13-mediated plant virus resistance is that it might lead to the emergence of new viruses (Ali et al. 2018).

14.5 Future Prospects

Cas13 structural and functional variants with a single protein effector module enable their structure-guided engineering for future applications in RNA targeting of plant viral pathogens, cytoplasmic, non-coding nuclear transcripts, specific isoforms, and pre-mRNA, tracking and editing with string efficiency, robustness, versatile specificity, less non-sensitivity to RNA secondary structures, low price tag, and affluence of maneuverability. Protein-RNA binding with probes having desired tags, editing of RNA metabolism machinery and RNA sequence manipulations, simultaneously, by fusing diverse effectors to respective variants and targeting their mRNAs, can lead to knocking down of genes, with better specificity than RNAi/CRISPRi.

References

Abudayyeh OO, Gootenberg JS, Essletzbichler P, Han S, Joung J, Belanto JJ et al (2017) RNA targeting with CRISPR-Cas13. Nature 550(7675):280–284. https://doi.org/10.1038/nature24049

Abudayyeh OO, Gootenberg JS, Konermann S, Joung J, Slaymaker IM, Cox DB, Shmakov S, Makarova KS, Semenova E, Minakhin L, Severinov K, Regev A, Lander ES, Koonin EV, Zhang F (2016) C2c2 is a single-component programmable RNA-guided RNA-targeting CRISPR effector. Science 353(6299):aaf5573. https://doi.org/10.1126/science.aaf5573

Abudayyeh OO, Gootenberg JS, Kellner MJ, Zhang F (2019a) Nucleic acid detection of plant genes using CRISPR-Cas13. CRISPR J 2(3):165–171. https://doi.org/10.1089/crispr.2019.0011

Abudayyeh OO, Gootenberg JS, Franklin B, Koob J, Kellner MJ, Ladha A, Joung J, Kirchgatterer P, Cox DBT, Zhang F (2019b) A cytosine deaminase for programmable single-base RNA editing. Science 365(6451):382–386. https://doi.org/10.1126/science.aax7063

Ali Z, Mahas A, Mahfouz M (2018) CRISPR/Cas13 as a tool for RNA interference. Trends Plant Sci 23:374–378

Aman R, Ali Z, Butt H et al (2018a) RNA virus interference via CRISPR/Cas13a system in plants. Genome Biol 19(1):1–9. https://doi.org/10.1186/s13059-017-1381-1

Aman R, Mahas A, Butt H, Aljedaani F, Mahfouz M (2018b) Engineering RNA virus interference via the CRISPR/Cas13 machinery in Arabidopsis. Viruses 10(12):732. https://doi.org/10.3390/v10120732

Amitai G, Sorek R (2016) CRISPR-Cas adaptation: insights into the mechanism of action. Nat Rev Microbiol 14(2):67–76. https://doi.org/10.1038/nrmicro.2015.14

Anantharaman V, Makarova KS, Burroughs AM, Koonin EV, Aravind L (2013) Comprehensive analysis of the HEPN superfamily: identification of novel roles in intra-genomic conflicts, defense, pathogenesis and RNA processing. Biol Direct 8:15. https://doi.org/10.1186/1745-6150-8-15

Batra R, Nelles DA, Pirie E et al (2017) Elimination of toxic microsatellite repeat expansion RNA by RNA-targeting Cas9. Cell 170(5):899–912, e10. https://doi.org/10.1016/j.cell.2017.07.010
Baulcombe DC (1996) RNA as a target and an initiator of post-transcriptional gene silencing in transgenic plants. Plant Mol Biol 32(1–2):79–88. https://doi.org/10.1007/bf00039378
Ben-Ari Y, Brody Y, Kinor N et al (2010) The life of an mRNA in space and time. J Cell Sci 123(Pt 10):1761–1774. https://doi.org/10.1242/jcs.062638
Benda C, Ebert J, Scheltema RA, Schiller HB, Baumgärtner M, Bonneau F, Mann M, Conti E (2014) Structural model of a CRISPR RNA-silencing complex reveals the RNA-target cleavage activity in Cmr4. Mol Cell 56(1):43–54. https://doi.org/10.1016/j.molcel.2014.09.002
Biswas A, Fineran PC, Brown CM (2014) Accurate computational prediction of the transcribed strand of CRISPR non-coding RNAs. Bioinformatics 30:1805–1813
Boch J, Bonas U (2010) Xanthomonas AvrBs3 family-type III effectors: discovery and function. Annu Rev Phytopathol 48:419–436. https://doi.org/10.1146/annurev-phyto-080508-081936
Bortesi L, Fischer R (2015) The CRISPR/Cas9 system for plant genome editing and beyond. Biotechnol Adv 33:41–52. https://doi.org/10.1016/j.biotechadv.2014.12.006
Brooks C, Nekrasov V, Lipppman ZB, Van Eck J (2014) Efficient gene editing in tomato in the first generation using the clustered regularly interspaced short palindromic repeats/CRISPR-associated9 system. Plant Physiol 166:1292–1297. https://doi.org/10.1104/pp.114.247577
Burstein D, Harrington LB, Strutt SC, Probst AJ, Anantharaman K, Thomas BC et al (2017) New CRISPR-Cas systems from uncultivated microbes. Nature 542:237–241
Cai CQ, Doyon Y, Ainley WM, Miller JC, Dekelver RC, Moehle EA, Rock JM, Lee YL, Garrison R, Schulenberg L et al (2009) Targeted transgene integration in plant cells using designed zinc finger nucleases. Plant Mol Biol 69:699–709. https://doi.org/10.1007/s11103-008-9449-7
Che P, Anand A, Wu E et al (2018) Developing a flexible, high-efficiency Agrobacterium-mediated sorghum transformation system with broad application. Plant Biotechnol J 16:1388–1395. https://doi.org/10.1111/pbi.12879
Chen M, Ma Z, Wu X et al (2017) A molecular beacon-based approach for live-cell imaging of RNA transcripts with minimal target engineering at the single-molecule level. Sci Rep 7:1550. https://doi.org/10.1038/s41598-017-01740-1
Chen S, Lee B, Lee AYF et al (2016) Highly efficient mouse genome editing by CRISPR ribonucleoprotein electroporation of zygotes. J Biol Chem 291:14457–14467. https://doi.org/10.1074/jbc.M116.733154
Chylinski K, Makarova KS, Charpentier E, Koonin EV (2014) Classification and evolution of type II CRISPR-Cas systems. Nucl Acids Res 42:6091–6105
Cox DBT, Gootenberg JS, Abudayyeh OO, Franklin B, Kellner MJ, Joung J, Zhang F (2017) RNA editing with CRISPR-Cas13. Science 358(6366):1019–1027. https://doi.org/10.1126/science.aaq0180
Davis BJ, O'Connell MR (2020) Put on your para-spectacles: the development of optimized CRISPR-Cas13-based approaches to image RNA dynamics in real time. Mol Cell 77(2):207–209
De Francesco L (2012) Move over ZFNs. Nat Biotechnol 30(1):112. https://doi.org/10.1038/nbt.1935
de Pater S, Neuteboom LW, Pinas JE, Hooykaas PJ, van der Zaal BJ (2009) ZFN-induced mutagenesis and gene-targeting in Arabidopsis through Agrobacterium-mediated floral dip transformation. Plant Biotechnol J 7:821–835. https://doi.org/10.1111/j.1467-7652.2009.00446.x
Doudna JA, Charpentier E (2014) The new frontier of genome engineering with CRISPR-Cas9. Science 346(6213):1258096. https://doi.org/10.1126/science.1258096
Dreissig S, Schiml S, Schindele P, Weiss O, RuttenT Schubert V, Gladilin E et al (2017) Live-cell CRISPR imaging in plants reveals dynamic telomere movements. Plant J 91:565–573. https://doi.org/10.1111/tpj.13601
Duan YB, Li J, Qin RY et al (2016) Identification of a regulatory element responsible for salt induction of rice OsRAV2 through ex situ and in situ promoter analysis. Plant Mol Biol 90:49–62. https://doi.org/10.1007/s11103-015-0393-z

East-Seletsky A, O'Connell MR, Burstein D, Knott GJ, Doudna JA (2017) RNA targeting by functionally orthogonal type VI-A CRISPR-Cas enzymes. Mol Cell 66:373–383, e373. https://doi.org/10.1016/j.molcel.2017.04.008

East-Seletsky A, O'Connell MR, Knight SC, Burstein D, Cate JH, Tjian R, Doudna JA (2016) Two distinct RNase activities of CRISPR-C2c2 enable guide-RNA processing and RNA detection. Nature 538(7624):270–273. https://doi.org/10.1038/nature19802

Femino AM, Fogarty K, Lifshitz LM, Carrington W, Singer RH (2003) Visualization of single molecules of mRNA in situ. Methods Enzym 361:245–304. https://doi.org/:10.1016/s0076-6879(03)61015-3

Feng Z, Mao Y, Xu N, Zhang B, Wei P et al (2014) Multigeneration analysis reveals the inheritance, specificity, and patterns of CRISPR/Cas-induced gene modifications in Arabidopsis. PNAS 111:4632–4637. https://doi.org/10.1073/pnas.1400822111

Feng Z, Zhang B, Ding W, Liu X, Yang DL, Wei P, Cao F, Zhu S, Zhang F, Mao Y, Zhu JK (2013) Efficient genome editing in plants using a CRISPR/Cas system. Cell Res 23(10):1229–1232. https://doi.org/10.1038/cr.2013.114

Filonov GS, Moon JD, Svensen N, Jaffrey SR (2014) Broccoli: rapid selection of an RNA mimic of green fluorescent protein by fluorescence-based selection and directed evolution. J Am Chem Soc 136(46):16299–16308. https://doi.org/10.1021/ja508478x

Gao X, Tsang JC, Gaba F, Wu D, Lu L, Liu P (2014) Comparison of TALE designer transcription factors and the CRISPR/dCas9 in regulation of gene expression by targeting enhancers. Nucleic Acids Res 42:e155. https://doi.org/10.1093/nar/gku836

Gjaltema RAF, Schulz EG (2018) CRISPR/dCas9 switch systems for temporal transcriptional control. In: Epigenome editing. Humana Press, New York, NY, pp 167–185. https://doi.org/10.1007/978-1-4939-7774-1_8

Grynberg M, Erlandsen H, Godzik A (2003) HEPN: a common domain in bacterial drug resistance and human neurodegenerative proteins. Trends Biochem Sci 28:224–226

Gootenberg JS, Abudayyeh OO, Lee JW et al (2017) Nucleic acid detection with CRISPR-Cas13a/C2c2. Science 356(6336):438–442. https://doi.org/10.1126/science.aam9321

Guo Y, Xu Q, Canzio D, Shou J, Li J, Gorkin DU, Jung I et al (2015) CRISPR inversion of CTCF sites alters genome topology and enhancer/promoter function. Cell 162:900–910. https://doi.org/10.1016/j.cell.2015.07.038

Gupta RM, Kiran M (2014) Expanding the genetic editing tool kit: ZFNs, TALENs, and CRISPR-Cas9. J Clin Invest 124(10):4154–4161. https://doi.org/10.1172/JCI72992

Gurushidze M, Hensel G, Hiekel S, Schedel S, Valkov V, Kumlehn J (2014) True-breeding targeted gene knock-out in barley using designer TALE-nuclease in haploid cells. PLoS One 9(3):e92046. https://doi.org/10.1371/journal.pone.0092046

Haun W, Coffman A, Clasen BM et al (2014) Improved soybean oil quality by targeted mutagenesis of the fatty acid desaturase 2 gene family. Plant Biotechnol J 2:934–940. https://doi.org/10.1111/pbi.12201

Hilton IB, D'Ippolito AM, Vockley CM, Thakore PI, Crawford GE, Reddy TE, Gersbach CA (2015) Epigenome editing by a CRISPR-Cas9-based acetyltransferase activates genes from promoters and enhancers. Nat Biotechnol 33:510–517. https://doi.org/10.1038/nbt.3199

Horvath P, Barrangou R (2010) CRISPR/Cas, the immune system of bacteria and archaea. Science 327:167–170. https://doi.org/10.1126/science.1179555

Hsu PD, Lander ES, Zhang F (2014) Development and applications of CRISPR-Cas9 for genome engineering. Cell 157(6):1262–1278. https://doi.org/10.1016/j.cell.2014.05.010

Hu X, Wang C, Fu Y, Liu Q, Jiao X, Wang K (2016) Expanding the range of CRISPR/Cas9 genome editing in rice. Mol Plant 9:943–945. https://doi.org/10.1016/j.molp.2016.03.003

Iranzo J, Lobkovsky AE, Wolf YI, Koonin EV (2015) Immunity, suicide or both? Ecological determinants for the combined evolution of anti-pathogen defense systems. BMC Evol Biol 15:43

Ivancic-Bace I, Cass SD, Wearne SJ, Bolt EL (2015) Different genome stability proteins underpin primed and naive adaptation in E. coli CRISPR-Cas immunity. Nucleic Acids Res 43(22):10821–10830. https://doi.org/10.1093/nar/gkv1213

Jaganathan D, Ramasamy K, Sellamuthu G et al (2018) CRISPR for crop improvement: an update review. Front Plant Sci 9:1–17. https://doi.org/10.3389/fpls.2018.00985

Jiang W, Bikard D, Cox D, Zhang F, Marraffini LA (2013) RNA-guided editing of bacterial genomes using CRISPR-Cas systems. Nat Biotechnol 31:233–239. https://doi.org/10.1038/nbt.2508

Jiang F, Doudna JA (2015) The structural biology of CRISPR-Cas systems. Curr Opin Struct Biol 30:100–111. https://doi.org/10.1016/j.sbi.2015.02.002

Jiang W, Yang B, Weeks DP (2014) Efficient CRISPR/Cas9-Mediated gene editing in Arabidopsis thaliana and inheritance of modified genes in the T2 and T3 generations. PLoS One 9(6):e99225. https://doi.org/10.1371/journal.pone.0099225

Jinek M, Chylinski K, Fonfara I, Hauer M, Doudna JA, Charpentier E (2012) A programmable dual-RNA–guided DNA endonuclease in adaptive bacterial immunity. Science 337(6096):816–821. https://doi.org/10.1126/science.1225829

Jinek M, Jiang F, Taylor DW et al (2014) Structures of Cas9 endonucleases reveal RNA-mediated conformational activation. Science 80(343):1–28. https://doi.org/10.1126/science.1247997

Jung C, Capistrano-Gossmann G, Braatz J, Sashidhar N, Melzer S (2018) Recent developments in genome editing and applications in plant breeding. Plant Breed 137:1–9. https://doi.org/10.1111/pbr.12526

Kazama T, Okuno M, Watari Y et al (2019) Curing cytoplasmic male sterility via TALEN-mediated mitochondrial genome editing. Nat Plants 5:722–730. https://doi.org/10.1038/s41477-019-0459-z

Kazlauskiene M, Kostiuk G, Venclovas Č et al (2017) A cyclic oligonucleotide signaling pathway in type III CRISPR-Cas systems. Science 357(80–):605–609. https://doi.org/10.1126/science.aao0100

Kieper SN, Almendros C, Behler J, McKenzie RE, Nobrega FL, Haagsma AC, Vink JNA, Hess WR, Brouns SJJ (2018) Cas4 facilitates PAM-compatible spacer selection during CRISPR adaptation. Cell Rep 22(13):3377–3384. https://doi.org/10.1016/j.celrep.2018.02.103

Kim D, Alptekin B, Budak H (2018) CRISPR/Cas9 genome editing in wheat. Funct Integr Genomics 18:31–41. https://doi.org/10.1007/s10142-017-0572-x

Kim YG, Cha J, Chandrasegaran S (1996) Hybrid restriction enzymes: zinc finger fusions to Fok I cleavage domain. PNAS 93:1156–1160. https://doi.org/10.1073/pnas.93.3.1156

Knott GJ, East-Seletsky A, Cofsky JC, Holton JM, Charles E, O'Connell MR, Doudna JA (2017) Guide-bound structures of an RNA-targeting A-cleaving CRISPR-Cas13a enzyme. Nat Struct Mol Biol 24(10):825. https://doi.org/10.1038/nsmb.3466

Konermann S, Lotfy P, Brideau NJ, Oki J, Shokhirev MN, Hsu PD (2018) Transcriptome engineering with RNA-targeting type VI-D CRISPR Effectors. Cell 173(3):665–676, e614. https://doi.org/10.1016/j.cell.2018.02.033

Koonin EV, Krupovic M (2015) Evolution of adaptive immunity from transposable elements combined with innate immune systems. Nat Rev Genet 16:184–192

Koonin EV, Makarova KS, Zhang F (2017) Diversity, classification and evolution of CRISPR-Cas systems. Curr Opin Microbiol 37:67–78. https://doi.org/10.1016/j.mib.2017.05.008

Koonin EV, Dolja VV, Krupovic M (2015) Origins and evolution of viruses of eukaryotes: the ultimate modularity. Virology 479–480:2–25

Krupovic M, Makarova KS, Forterre P, Prangishvili D, Koonin EV (2014) Casposons: a new superfamily of self-synthesizing DNA transposons at the origin of prokaryotic CRISPR-Cas immunity. BMC Biol 12:36

Kunne T, Kieper SN, Bannenberg JW, Vogel AI, Miellet WR, Klein M, Depken M, Suarez-Diez M, Brouns SJ (2016) Cas3-derived target DNA degradation fragments fuel primed CRISPR adaptation. Mol Cell 63(5):852–864. https://doi.org/10.1016/j.molcel.2016.07.011

Larson DR, Zenklusen D, Wu B, Chao JA, Singer RH (2011) Real-time observation of transcription initiation and elongation on an endogenous yeast gene. Science 332(6028):475–478. https://doi.org/10.1126/science.1202142

Lee CM, Cradick TJ, Bao G (2016) The Neisseria meningitidis CRISPR-Cas9 system enables specific genome editing in mammalian cells. Mol Ther 24:645–654. https://doi.org/10.1038/mt.2016.8

Lee H, Zhou Y, Taylor DW, Sashital DG (2018) Cas4-dependent prespacer processing ensures high-fidelity programming of CRISPR arrays. Mol Cell 70(1):48–59, e45. https://doi.org/10.1016/j.molcel.2018.03.003

Levy A, Goren MG, Yosef I, Auster O, Manor M, Amitai G, Edgar R, Qimron U, Sorek R (2015) CRISPR adaptation biases explain preference for acquisition of foreign DNA. Nature 520(7548):505–510. https://doi.org/10.1038/nature14302

Li JF, Norville JE, Aach J, McCormack M, Zhang D, Bush J et al (2013) Multiplex and homologous recombination-mediated genome editing in Arabidopsis and Nicotiana benthamiana using guide RNA and Cas9. Nat Biotechnol 31:688–691. https://doi.org/10.1038/nbt.2654

Liang Z, Chen K, Li T, Zhang Y, Wang Y, Zhao Q, Liu J et al (2017) ARTICLE efficient DNA-free genome editing of bread wheat using CRISPR/Cas9 ribonucleoprotein complexes. Nat Publ Gr 8:1–5. https://doi.org/10.1038/ncomms14261

Lloyd A, Plaisier CL, Carroll D et al (2005) Targeted mutagenesis using zinc-finger nucleases in Arabidopsis. PNAS USA 102:2232–2237. https://doi.org/10.1073/pnas.0409339102

Liu E, Zeng S, Zhu S et al (2019) Favorable alleles of GRAIN-FILLING RATE1 increase the grain-filling rate and yield of rice. Plant Physiol 181:1207–1222. https://doi.org/10.1104/pp.19.00413

Liu L, Li X, Ma J, Li Z, You L, Wang J, Wang M, Zhang X, Wang Y (2017a) The molecular architecture for RNA-guided RNA cleavage by Cas13a. Cell 170(4):714–726, e710. https://doi.org/10.1016/j.cell.2017.06.050

Liu L, Li X, Wang J, Wang M, Chen P, Yin M, Li J, Sheng G, Wang Y (2017b) Two distant catalytic sites are responsible for C2c2 RNase activities. Cell 168(1–2): 121–134, e112. https://doi.org/10.1016/j.cell.2016.12.031

Lor VS, Starker CG, Voytas DF, Weiss D, Olszewski NE (2014) Targeted mutagenesis of the tomato PROCERA gene using transcription activator-like effector nucleases. Plant Physiol 166:1288–1291. https://doi.org/10.1104/pp.114.247593

Lowder L, Malzahn A, Qi Y (2016) Rapid evolution of manifold CRISPR systems for plant genome editing. Front Plant Sci 7:1–12. https://doi.org/10.3389/fpls.2016.01683

Lowder LG, Paul JW, Qi Y (2017a) Multiplexed transcriptional activation or repression in plants using CRISPR-dCas9-based systems. Plant gene regulatory networks. Humana Press, New York, NY, pp 167–184. https://doi.org/10.1007/978-1-4939-7125-1_12

Lowder LG, Zhou J, Zhang Y, Malzahn A, Zhong Z, Hsieh T-F, Voytas DF et al (2017b) Robust transcriptional activation in plants using multiplexed CRISPR-Act2.0 and mTALE-Act systems. Mol Plant 11:245–256. https://doi.org/10.1016/j.molp.2017.11.010

Macovei A, Sevilla NR, Cantos C et al (2018) Novel alleles of rice eIF4G generated by CRISPR/Cas9-targeted mutagenesis confer resistance to Rice tungro spherical virus. Plant Biotechnol J 16:1918–1927. https://doi.org/10.1111/pbi.12927

Maeder ML, Thibodeau-Beganny S, Osiak A, Wright DA, Anthony RM, Eichtinger M, Jiang T, Foley JE, Winfrey RJ, Townsend JA, Unger-Wallace E, Sander JD, Müller-Lerch F, Fu F, Pearlberg J, Göbel C, Dassie JP, Pruett-Miller SM, Porteus MH, Sgroi DC, Iafrate AJ, Dobbs D, McCray PB Jr, Cathomen T, Voytas DF, Joung JK (2008) Rapid "open-source" engineering of customized zinc-finger nucleases for highly efficient gene modification. Mol Cell 25:31(2):294–301. https://doi.org/10.1016/j.molcel.2008.06.016

Mahas A, Aman R, Mahfouz M (2019) CRISPR-Cas13d mediates robust RNA virus interference in plants. Genome Biol 20:263. https://doi.org/10.1186/s13059-019-1881-2

Mahas A, Mahfouz M (2018) Engineering virus resistance via CRISPR-Cas systems. Curr Opin Virol 32:1–8

Mahfouz MM, Li L (2011) TALE nucleases and next generation GM crops. GM Crops 2:99–103. https://doi.org/10.4161/gmcr.2.2.17254
Majumdar S, Zhao P, Pfister NT, Compton M, Olson S, Glover CV 3rd, Wells L, Graveley BR, Terns RM, Terns MP (2015) Three CRISPR-Cas immune effector complexes coexist in Pyrococcus furiosus. RNA 21:1147–1158
Makarova KS, Anantharaman V, Aravind L, Koonin EV (2012) Live virus-free or die: coupling of antivirus immunity and programmed suicide or dormancy in prokaryotes. Biol Direct 7:40. https://doi.org/10.1186/1745-6150-7-40
Makarova KS, Anantharaman V, Grishin NV, Koonin EV, Aravind L (2014) CARF and WYL domains: ligand-binding regulators of prokaryotic defense systems. Front Genet 5:102
Makarova KS, Haft DH, Barrangou R, Brouns SJ, Charpentier E, Horvath P, Moineau S, Mojica FJ, Wolf YI, Yakunin AF et al (2011) Evolution and classification of the CRISPR-Cas systems. Nat Rev Microbiol 9:467–477
Makarova KS, Wolf YI, Alkhnbashi OS, Costa F, Shah SA, Saunders SJ et al (2015) An updated evolutionary classification of CRISPR-Cas systems. Nat Rev Microbiol 13:722–736
Makarova KS, Wolf YI, Koonin EV (2013) The basic building blocks and evolution of CRISPR-cas systems. Biochem Soc Trans 41:1392–1400
Mao Y, Zhang H, Xu N, Zhang B, Gou F, Zhu JK (2013) Application of the CRISPR-Cas system for efficient genome engineering in plants. Mol Plant 6:2008–2011. https://doi.org/10.1093/mp/sst121
Marraffini LA, Sontheimer EJ (2008) CRISPR interference limits horizontal gene transfer in staphylococci by targeting DNA. Science 322:1843–1845. https://doi.org/10.1126/science.1165771
McGinn J, Marraffini LA (2016) CRISPR-Cas systems optimize their immune response by specifying the site of spacer integration. Mol Cell 64(3):616–623. https://doi.org/10.1016/j.molcel.2016.08.038
McGinn J, Marraffini LA (2019) Molecular mechanisms of CRISPR-Cas spacer acquisition. Nat Rev Microbiol 17(1):7–12. https://doi.org/10.1038/s41579-0180071-7
Meeske AJ, Marraffini LA (2018) RNA guide complementarity prevents self-targeting in type VI CRISPR systems. Mol Cell 71:791–801. e3. https://doi.org/10.1016/j.molcel.2018.07.013
Miao J, Guo D, Zhang J, Huang Q, Qin G, Zhang X et al (2013) Targeted mutagenesis in rice using CRISPR-Cas system. Cell Res 23:1233–1236. https://doi.org/10.1038/cr.2013.123
Mishra R, Zhao K (2018) Genome editing technologies and their applications in crop improvement. Plant Biotechnol Rep 12:57–68. https://doi.org/10.1007/s11816-018-0472-0
Modell JW, Jiang W, Marraffini LA (2017) CRISPR-Cas systems exploit viral DNA injection to establish and maintain adaptive immunity. Nature 544(7648):101–104. https://doi.org/10.1038/nature21719
Moradpour M, Abdulah SNA (2020) CRISPR/dCas9 platforms in plants: strategies and applications beyond genome editing. Plant Biotechnol J 18:32–44. https://doi.org/10.1111/pbi.13232
Mulepati S, Bailey S, Directorate S (2015) Bound to a ssDNA target 345:1479–1484. https://doi.org/10.1126/science.1256996.Crystal
Nekrasov V, Staskawicz B, Weigel D, Jones JD, Kamoun S (2013) Targeted mutagenesis in the model plant Nicotiana benthamiana using Cas9 RNA-guided endonuclease. Nat Biotechnol 31:691–693. https://doi.org/10.1038/nbt.2655
Nelles DA, Fang MY, O'Connell MR et al (2016) Programmable RNA tracking in live cells with CRISPR/Cas9. Cell 165(2):488–496. https://doi.org/10.1016/j.cell.2016.02.054
Nicaise V (2014) Crop immunity against viruses: outcomes and future challenges. Front Plant Sci 5:660. https://doi.org/10.3389/fpls.2014.00660
Niewoehner O, Garcia-Doval C, Rostøl JT et al (2017) Type III CRISPR-Cas systems produce cyclic oligoadenylate second messengers. Nature 548:543–548. https://doi.org/10.1038/nature23467
Niewoehner O, Jinek M (2016) Structural basis for the endoribonuclease activity of the type III-A CRISPR associated protein Csm6. RNA 22:318–329

Nishimasu H, Ran FA, Hsu PD, Konermann S, Shehata SI, Dohmae N et al (2014) Crystal structure of Cas9 in complex with guide RNA and target DNA. Cell 156:935–949. https://doi.org/10.1016/j.cell.2014.02.001

Norais C, Moisan A, Gaspin C, Clouet-d'Orval B (2013) Diversity of CRISPR systems in the euryarchaeal Pyrococcales. RNA Biol 10:659–670

Nunez JK, Harrington LB, Kranzusch PJ, Engelman AN, Doudna JA (2015a) Foreign DNA capture during CRISPR-Cas adaptive immunity. Nature 527(7579):535–538. https://doi.org/10.1038/nature15760

Nunez JK, Kranzusch PJ, Noeske J, Wright AV, Davies CW, Doudna JA (2014) Cas1-Cas2 complex formation mediates spacer acquisition during CRISPR-Cas adaptive immunity. Nat Struct Mol Biol 21(6):528–534. https://doi.org/10.1038/nsmb.2820

Nunez JK, Lee AS, Engelman A, Doudna JA (2015b) Integrase-mediated spacer acquisition during CRISPR-Cas adaptive immunity. Nature 519(7542):193–198. https://doi.org/10.1038/nature14237

O'Connell M (2019) Molecular mechanisms of RNA-targeting by Cas13-containing type VI CRISPR-Cas systems. J Mol Biol 431(1):66–87

Oliva R, Ji C, Atienza-Grande G et al (2019) Broad-spectrum resistance to bacterial blight in rice using genome editing. Nat Biotechnol 37:1344–1350. https://doi.org/10.1038/s41587-019-0267-z

Osakabe K, Osakabe Y, Toki S (2010) Site-directed mutagenesis in Arabidopsis using custom-designed zinc finger nucleases. Proc Natl Acad Sci USA107:12034–12039. https://doi.org/10.1073/pnas.1000234107

Pacher M, Puchta H (2017) From classical mutagenesis to nuclease-based breeding-directing natural DNA repair for a natural end-product. Plant J 90:819–833. https://doi.org/10.1111/tpj.13469

Paige JS, Wu KY, Jaffrey SR (2011) RNA mimics of green fluorescent protein. Science 333(6042):642–646. https://doi.org/10.1126/science.1207339

Pardo B, Gómez-González B, Aguilera A (2009) DNA repair in mammalian cells. Cell Mol Life Sci 66:1039–1056. https://doi.org/10.1007/s00018-009-8740-3

Petolino JF, Worden A, Curlee K, Connell J, Strange Moynahan TL, Larsen C, Russell S (2010) Zinc finger nuclease-mediated transgene deletion. Plant Mol Biol 73:617–628. https://doi.org/10.1007/s11103-010-9641-4

Prado JR, Segers G, Voelker T, Carson D, Dobert R et al (2014) Genetically engineered crops: from idea to product. Annu Rev Plant Biol 65:769–790. https://doi.org/10.1146/annurev-arplant-050213-040039

Qi Y, Li X, Zhang Y, Starker CG, Baltes NJ, Zhang F, Sander JD, Reyon D, Joung KJ, Voytas DF (2013) Targeted deletion and inversion of tandemly arrayed genes in Arabidopsis thaliana somatic cells using zinc finger nucleases. G3 3:1707–1715. https://doi.org/10.1534/g3.113.006270

Raj A, van den Bogaard P, Rifkin SA, van Oudenaarden A, Tyagi S (2008) Imaging individual mRNA molecules using multiple singly labeled probes. Nat Methods 5(10):877–879. https://doi.org/10.1038/nmeth.1253

Ran FA, Cong L, Yan WX, Scott DA, Gootenberg JS, Kriz AJ et al (2015) In vivo genome editing using Staphylococcus aureus Cas9. Nature 520:186–191. https://doi.org/10.1038/nature14299

Ricroch A, Clairand P, Harwood W (2017) Use of CRISPR systems in plant genome editing: toward new opportunities in agriculture. Emerg Top Life Sci 1:169–182. https://doi.org/10.1042/etls20170085

Sander JD (2019) Gene editing in sorghum through agrobacterium. Methods Mol Biol 1931:155–168. https://doi.org/10.1007/978-1-4939-9039-9_11

Schiml S, Fauser F, Puchta H (2014) The CRISPR/Cas system can be used as nuclease for in planta gene targeting and as paired nickases for directed mutagenesis in Arabidopsis resulting in heritable progeny. Plant J80:1139–1150. https://doi.org/10.1111/tpj.12704

Semenova E, Jore MM, Datsenko KA, Semenova A, Westra ER, Wanner B, van der Oost J, Brouns SJ, Severinov K (2011) Interference by clustered regularly interspaced short palindromic repeat

(CRISPR) RNA is governed by a seed sequence. Proc Natl Acad Sci USA 108(25):10098–10103. https://doi.org/10.1073/pnas.1104144108
Severinov K, Zhang F, Wolf Y et al (2017) Novel CRISPR enzymes and systems. U.S. patent application no. US15/ 482,603
Shan Q, Wang Y, Chen K et al (2013a) Rapid and efficient gene modification in rice and Brachypodium using TALENs. Mol Plant 6:1365–1368. https://doi.org/10.1093/mp/sss162
Shan Q, Wang Y, Li J, Zhang Y, Chen K et al (2013b) Targeted genome modification of crop plants using a CRISPR-Cas system. Nat Biotechnol 31:686–688. https://doi.org/10.1038/nbt.2650
Sheppard NF, Glover CV, Terns RM, Terns MP (2016) The CRISPR-associated Csx1 protein of Pyrococcus furiosus is an adenosine-specific endoribonuclease. RNA 22:216–224
Shi J, Gao H, Wang H et al (2017) ARGOS8 variants generated by CRISPR-Cas9 improve maize grain yield under field drought stress conditions. Plant Biotechnol J 15:207–216. https://doi.org/10.1111/pbi.12603
Shiimori M, Garrett SC, Graveley BR, Terns MP (2018) Cas4 Nucleases define the PAM, length, and orientation of DNA fragments integrated at CRISPR Loci. Mol. Cell 70(5):814–824, e816. https://doi.org/10.1016/j.molcel.2018.05.002
Shmakov S, Abudayyeh OO, Makarova KS, Wolf YI, Gootenberg JS, Semenova E, Minakhin L, Joung J, Konermann S, Severinov K, Zhang F, Koonin EV (2015) Discovery and functional characterization of diverse class 2 CRISPR-Cas systems. Mol Cell 60(3):385–397. https://doi.org/10.1016/j.molcel.2015.10.008
Shmakov S, Smargon A, Scott D, Cox D, Pyzocha N, Yan W et al (2017) Diversity and evolution of class 2 CRISPR-Cas systems. Nat Rev Microbiol 15(3):169–182. https://doi.org/10.1038/nrmicro.2016.184
Shukla VK, Doyon Y, Miller JC, DeKelver RC, Moehle EA, Worden SE, Mitchell JC, Arnold NL, Gopalan S, Meng X et al (2009) Precise genome modification in the crop species Zea mays using zinc-finger nucleases. Nature 459:437–441. https://doi.org/10.1038/nature07992
Silas S, Lucas-Elio P, Jackson SA, Aroca-Crevillen A, Hansen LL, Fineran PC et al (2017) Type III CRISPR-Cas systems can provide redundancy to counteract viral escape from type I systems. eLife 6:e27601. https://doi.org/10.7554/eLife.27601
Silas S, Mohr G, Sidote DJ, Markham LM, Sanchez-Amat A, Bhaya D, Lambowitz AM et al. (2016) Direct CRISPR spacer acquisition from RNA by a natural reverse transcriptase-Cas1 fusion protein. Science 351:aad4234. https://doi.org/10.1126/science.aad4234
Simon-Mateo C, Garcia JA (2011) Antiviral strategies in plants based on RNA silencing. Biochim Biophys Acta 1809(11–12):722–731. https://doi.org/10.1016/j.bbagrm.2011.05.011
Slaymaker IM, Mesa P, Kellner MJ, Kannan S, Brignole E, Koob J et al (2019) High-resolution structure of Cas13b and biochemical characterization of RNA targeting and cleavage. Cell Rep 26(13):3741–3751, e5
Smargon AA, Cox DB, Pyzocha NK, Zheng K, Slaymaker IM, Gootenberg JS, Abudayyeh OA, Essletzbichler P, Shmakov S, Makarova KS, Koonin EV, Zhang F (2017) Cas13b is a type VI-B CRISPR-associated RNA-guided RNase differentially regulated by accessory proteins Csx27 and Csx28. Mol Cell 65(4):618–630. https://doi.org/10.1016/j.molcel.2016.12.023
Sorek R, Lawrence CM, Wiedenheft B (2013) CRISPR-mediated adaptive immune systems in bacteria and archaea. Annu Rev Biochem 82:237–266. https://doi.org/10.1146/annurev-biochem-072911-172315
Sprink T, Metje J, Hartung F (2015) Plant genome editing by novel tools: TALEN and other sequence specific nucleases. Curr Opin Biotechnol 32:47–53. https://doi.org/10.1016/j.copbio.2014.11.010
Tadele Z (2016) Mutagenesis and TILLING to dissect gene function in plants. Current Genom 17:499–508. https://doi.org/10.2174/1389202917666160520104158
Takeuchi N, Wolf YI, Makarova KS, Koonin EV (2012) Nature and intensity of selection pressure on CRISPR-associated genes. J Bacteriol 194:1216–1225
Tambe A, East-Seletsky A, Knott GJ, Doudna JA, O'Connell MR (2018) RNA binding and HEPN-nuclease activation are decoupled in CRISPR-Cas13a. Cell Rep 24(4):1025–1036. https://doi.org/10.1016/j.celrep.2018.06.105

Tamulaitis G, Kazlauskiene M, Manakova E, Venclovas Č, Nwokeoji AO, Dickman MJ, Horvath P, Siksnys V (2014) Programmable RNA shredding by the type III-A CRISPR-Cas system of streptococcus thermophilus. Mol Cell 56:506–517. https://doi.org/10.1016/j.molcel.2014.09.027

Tamulaitis G, Venclovas C, Siksnys V (2017) Type III CRISPR-Cas immunity: major differences brushed aside. Trends Microbiol 25(4):9–61. https://doi.org/10.1016/j.tim.2016.09.012

Tang Y, Fu Y (2018) Class 2 CRISPR/Cas: an expanding biotechnology toolbox for and beyond genome editing 06 Biological Sciences 0604 Genetics. Cell Biosci 8:1–13. https://doi.org/10.1186/s13578-018-0255-x

Tilman D, Balzer C, Hill J, Befort BL (2011) Global food demand and the sustainable intensification of agriculture. PNAS 108:20260–20264. https://doi.org/10.1073/pnas.1116437108

Toro N, Martinez-Abarca F, Gonzalez-Delgado A (2017) The reverse transcriptases associated with CRISPR-Cas systems. Sci Rep 7(708):9. https://doi.org/10.1038/s41598-017-07828-y

Tovkach A, Zeevi V, Tzfira T (2009) A toolbox and procedural notes for characterizing novel zinc finger nucleases for genome editing in plant cells. Plant J 57:747–757. https://doi.org/10.1111/j.1365-313X.2008.03718.x

Townsend JA, Wright DA, Winfrey RJ, Fu F, Maeder ML, Joung JK, Voytas DF (2009) High-frequency modification of plant genes using engineered zinc-finger nucleases. Nature 459:442–445. https://doi.org/10.1038/nature07845

Tyagi S, Kramer FR (1996) Molecular beacons: probes that fluoresce upon hybridization. Nat Biotechnol 14(3):303–308. https://doi.org/10.1038/nbt0396-303

Upadhyay SK, Kumar J, Alok A, Tuli R (2013) RNA-guided genome editing for target gene mutations in wheat. G3 (Bethesda) 3:2233–2238. https://doi.org/10.1534/g3.113.008847

Veillet F, Perrot L, Chauvin L, Kermarrec MP, Guyon-Debast A, Chauvin JE, Nogué F et al (2019) Transgene-free genome editing in tomato and potato plants using agrobacterium-mediated delivery of a CRISPR/Cas9 cytidine base editor. Int J Mol Sci 20:402. https://doi.org/10.3390/ijms20020402

Voytas DF (2013) Plant genome engineering with sequence-specific nucleases. Annu Rev Plant Biol 64:327–350. https://doi.org/10.1146/annurev-arplant-042811-105552

Wang F, Wang C, Liu P et al (2016) Enhanced rice blast resistance by CRISPR/ Cas9-targeted mutagenesis of the ERF transcription factor gene OsERF922. PLoS ONE 11:1–18. https://doi.org/10.1371/journal.pone.0154027

Wang J, Li J, Zhao H, Sheng G, Wang M, Yin M, Wang Y (2015) Structural and mechanistic basis of PAM-dependent spacer acquisition in CRISPR-Cas systems. Cell 163(4):840–853. https://doi.org/10.1016/j.cell.2015.10.008

Wang W, Hou J, Zheng N, Wang X, Zhang J (2019) Keeping our eyes on CRISPR: the "atlas" of gene editing. Cell Biol Toxicol 35:1–4. https://doi.org/10.1007/s10565-019-09480-w

Wang Y, Cheng X, Shan Q (2014) Simultaneous editing of three homoeoalleles in hexaploid bread wheat confers heritable resistance to powdery mildew. Nat Biotechnol 32:947–951. https://doi.org/10.1038/nbt.2969

Weinberger AD, Sun CL, Plucinski MM, Denef VJ, Thomas BC, Horvath P, Barrangou R, Gilmore MS, Getz WM, Banfield JF (2012) Persisting viral sequences shape microbial CRISPR-based immunity. PLoS Comput Biol 8(4):e1002475. https://doi.org/10.1371/journal.pcbi.1002475

Weinthal D, Tovkach A, Zeevi V, Tzfira T (2010) Genome editing in plant cells by zinc finger nucleases. Trends Plant Sci 15:308–321. https://doi.org/10.1016/j.tplants.2010.03.001

Wendt T, Holm PB, Starker CG, Christian M, Voytas DF et al (2013) TAL effector nucleases induce mutations at a pre-selected location in the genome of primary barley transformants. Plant Mol Biol. https://doi.org/10.1007/s11103-013-0078-4

Wiedenheft B, Sternberg SH, Doudna JA (2012) RNA-guided genetic silencing systems in bacteria and archaea. Nature 482:331–338. https://doi.org/10.1038/nature10886

Woo JW, Kim J, Kwon SIl, Corvalán C, Cho SW, Kim H, Kim SG et al (2015) DNA-free genome editing in plants with preassembled CRISPR-Cas9 ribonucleoproteins. Nat Biotechnol 33:1162–1164. https://doi.org/10.1038/nbt.3389

Wright AV, Liu JJ, Knott GJ, Doxzen KW, Nogales E, Doudna JA (2017) Structures of the CRISPR genome integration complex. Science 357(6356):1113–1118. https://doi.org/10.1126/science.aao0679

Wright DA, Townsend JA, Winfrey RJ et al (2005) High-frequency homologous recombination in plants mediated by zinc-finger nucleases. Plant J 44:693–705. https://doi.org/10.1111/j.1365-313X.2005.02551.x

Wu B, Chao JA, Singer RH (2012) Fluorescence fluctuation spectroscopy enables quantitative imaging of single mRNAs in living cells. Biophys J 20:102(12):2936–2944. https://doi.org/10.1016/j.bpj.2012.05.017

Xiao Y, Ng S, Nam KH, Ke A (2017) How type II CRISPR-Cas establish immunity through Cas1-Cas2-mediated spacer integration. Nature 550(7674):137–141. https://doi.org/10.1038/nature24020

Xie K, Yang Y (2013) RNA-Guided genome editing in plants using a CRISPR-Cas system. Mol Plant 6:1975–1983. https://doi.org/10.1093/mp/sst119

Xing HL, Dong L, Wang ZP, Zhang HY, Han CY, Liu B, Wang XC, Chen QJ (2014) A CRISPR/Cas9 toolkit for multiplex genome editing in plants. BMC Plant Biol 29(14):327. https://doi.org/10.1186/s12870-014-0327-y

Xue Y, Acar M (2018) Live-cell imaging of chromatin condensation dynamics by CRISPR. I Science 4:216–235. https://doi.org/10.1016/j.isci.2018.06.001

Yamano T, Nishimasu H, Zetsche B, Hirano H, Slaymaker IM, Li Y et al (2016) Crystal structure of Cpf1 in complex with guide RNA and target DNA. Cell 165:949–962. https://doi.org/10.1016/j.cell.2016.04.003

Yan WX, Chong S, Zhang H, Makarova KS, Koonin EV, Cheng DR, Scott DA (2018) Cas13d is a compact RNA-targeting type VI CRISPR effector positively modulated by a WYL-domain-containing accessory protein. Mol Cell 70(2):327–339, e5

Yang LZ, Wang Y, Li SQ, Yao RW, Luan PF, Wu H, Carmichael GG, Chen LL (2019) Dynamic imaging of RNA in living cells by CRISPR-Cas13 systems. Mol Cell 76(981–997):e987. https://doi.org/10.1016/j.molcel.2019.10.024

Yin K, Qiu JL (2019) Genome editing for plant disease resistance: applications and perspectives. Philos Trans R Soc B Biol Sci 374:20180322. https://doi.org/10.1098/rstb.2018.0322

Younis A, Siddique MI, Kim CK, Lim KB (2014) RNA Interference (RNAi) induced gene silencing: a promising approach of hi-tech plant breeding. Int J Biol Sci 10(10):1150–1158. https://doi.org/10.7150/ijbs.10452

Zetsche B, Gootenberg JS, Abudayyeh OO et al (2015) Cpf1 is a single RNA-guided endonuclease of a class 2 CRISPR-Cas system. Cell 163:759–771. https://doi.org/10.1016/j.cell.2015.09.038

Zetsche B, Heidenreich M, Mohanraju P, Fedorova I, Kneppers J, De Gennaro EM et al (2017) Multiplex gene editing by CRISPR-Cpf1 using a single crRNA array. Nat Biotechnol 35:31–34. https://doi.org/10.1038/nbt.3737

Zezulin A, Musunuru K (2018) Turning up the heat with therapeutic epigenome editing. Cell Stem Cell 22:10–11. https://doi.org/10.1016/j.stem.2017.12.013

Zhang A, Liu Y, Wang F et al (2019a) Enhanced rice salinity tolerance via CRISPR/Cas9-targeted mutagenesis of the OsRR22 gene. Mol Breed 39:47. https://doi.org/10.1007/s11032-019-0954-y

Zhang B, Ye W, Ye Y, Zhou H, Saeed AF, Chen J et al (2018a) Structural insights into Cas13b-guided CRISPR RNA maturation and recognition. Cell Res 28(12):1198

Zhang C, Konermann S, Brideau NJ, Lotfy P, Wu X, Novick SJ, Strutzenberg T, Griffin PR, Hsu PD, Lyumkis D (2018b) Structural basis for the RNA-guided ribonuclease activity of CRISPR-Cas13d. Cell 175(1):212–223, e17. https://doi.org/10.1016/j.cell.2018.09.001

Zhang F, Maeder ML, Unger-Wallace E, Hoshaw JP, Reyon D, Christian M, Li X, Pierick CJ, Dobbs D, Peterson T et al (2010) High frequency targeted mutagenesis in Arabidopsis thaliana using zinc finger nucleases. Proc Natl Acad Sci USA 107:12028–12033. https://doi.org/10.1073/pnas.0914991107

Zhang F, Wen Y, Guo X (2014) CRISPR/Cas9 for genome editing: Progress, implications and challenges. Hum Mol Genet 23:40–46. https://doi.org/10.1093/hmg/ddu125

Zhang H, Dong C, Li L, Wasney GA, Min J (2019b) Structural insights into the modulatory role of the accessory protein WYL1 in the type VI-D CRISPR-Cas system. Nucleic Acids Res 47(10):5420–5428

Zhang Y, Liang Z, Zong Y, Wang Y, Liu J, Chen K, Qiu JL et al (2016) Efficient and transgene-free genome editing in wheat through transient expression of CRISPR/Cas9 DNA or RNA. Nat Commun 7:12617. https://doi.org/10.1038/ncomms12617

Zhang Y, Massel K, Godwin ID, Gao C (2018c) Applications and potential of genome editing in crop improvement. Genome Biol 19(1):210. https://doi.org/10.1186/s13059-018-1586-y

Zhang Y, Zhang F, Li X (2013a) Transcription activator-like effector nucleases enable efficient plant genome engineering. Plant Physiol 161:20–27. https://doi.org/10.1104/pp.112.205179

Zhang Z, Xiang D, Heriyanto F, Gao Y, Qian Z, Wu WS (2013b) Dissecting the roles of miR-302/367 cluster in cellular reprogramming using TALE-based repressor and TALEN. Stem Cell Rep 1:218–225. https://doi.org/10.1016/j.stemcr.2013.07.002

Zhou HB, Liu B, Weeks DP, Spalding MH, Yang B (2014) Large chromosomal deletions and heritable small genetic changes induced by CRISPR/Cas9 in rice. Nucleic Acids Res 42:10903–10914. https://doi.org/10.1093/nar/gku806