CRISPR and Plant Functional Genomics

CRISPR is a crucial technology in plant physiology and molecular biology, resulting in more sustainable agricultural practices, including outcomes of better plant stress tolerance and crop improvement. *CRISPR and Plant Functional Genomics* explores ways to release the potential of plant functional genomics, one of the prevailing topics in plant biology and a critical technology for speed and precision crop breeding. This book presents achievements in plant functional genomics and features information on diverse applications using emerging CRISPR-based genome editing technologies producing high-yield, disease-resistant, and climate-smart crops. It also includes theories on organizing strategies for upgrading the CRISPR system to increase efficiency, avoid off-target effects, and produce transgene-free edited crops.

Features:

- Presents CRISPR-based technologies, releasing the potential of plant functional genomics
- Provides methods and applications of CRISPR/Cas-based plant genome editing technologies
- Summarizes achievements of speed and precision crop breeding using CRISPR-based technologies
- Illustrates strategies to upgrade the CRISPR system
- Supports the UN's sustainable development goals to develop future climate-resilient crops

CRISPR and Plant Functional Genomics provides extensive knowledge of CRISPR-based technologies and plant functional genomics and is an ideal reference for researchers, graduate students, and practitioners in the field of plant sciences as well as agronomy and agriculture.

CRISPR and Plant Functional Genomics

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Jen-Tsung Chen

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16 CRISPR/Cas13 for the Control of Plant Viruses

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LIST OF ABBREVIATIONS

ABE	adenine base editor
C2c2	class 2 candidate 2
C2c6	class 2 candidate 6
Cas	CRISPR-associated proteins
CBE	cytosine base editor
CRISPR	clustered regularly interspaced short palindromic repeats
crRNAs	CRISPR RNAs
DNA	deoxyribonucleic acid
DR	direct repeat
DSB	double-strand break
dsRNA	double-stranded RNA
HDR	homology-directed repair
HEPN	higher eukaryotes and prokaryotes nucleotide-binding domains
Lba	*Lachnospiraceae bacterium*
Lbu	*Leptotrichia buccalis*
Lsh	*Leptotrichia sharii*
Lwa	*Leptotrichia wadei*
Lse	*Listeria seeligeri*
nt	nucleotide
NTD	N-terminal domain
NUC	nuclease
PFS	protospacer flanking site
REC	recognition
RNA	ribonucleic acid
sgRNA	single guide RNA
ssRNA	single-stranded RNA
TMV	*Tobacco mosaic virus*
TuMV	*Turnip mosaic virus*
tracrRNAs	transactivating CRISPR RNAs

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16.1 INTRODUCTION

Viruses are among the most important pathogens in both animals and plants. These agents have RNA or DNA genomes, single or double-stranded, encoding some proteins responsible for their replication and transmission. Viruses can only replicate inside living cells of a host organism and their host range is usually relatively narrow [1].

Plant viruses have major implications on plant pathology and the study of virology, which dates back to early studies using *Tobacco mosaic virus* (TMV) which helped to understand the virus concept, uncovering chemical and physical characteristics of viruses in general [2]. Plant viruses can rapidly replicate and spread throughout a crop, being very difficult to monitor and control and, therefore, causing destructive diseases in many agricultural systems. These diseases can significantly reduce crop quality and yield, resulting in tremendous economic impacts all over the world and threatening food security and provision [3].

Unlike what happens with other plant disease-causing pathogens, there are no efficient chemical products that can eradicate a virus within a plant without disturbing host cells and the environment. Consequently, preventive sanitary measures, such as the use of viral-resistant plants, are usually the only options. Resistant plants were conventionally generated through a very time-consuming classical breeding process. However, nowadays, virus-resistant plants can be generated through molecular plant breeding, preventing and controlling viral diseases [4,5]. These molecular approaches can be based on genomic selection, molecular marker-assisted breeding, gene silencing, and pathogen-derived resistance, however, many setbacks have hampered their utility in agriculture. The major drawback is the fast adaptation and emergence of new viruses for which these techniques are not efficient enough. In addition, gene knockout in plants to prevent viral replication can compromise other desirable characteristics [3,6].

Over the past decade, a breakthrough has revolutionized plant breeding. The study of clustered regularly interspaced short palindromic repeats (CRISPR) and CRISPR-associated (Cas) protein systems allowed the development of a new technology that has opened up new horizons for plant breeding and improvement. The function of CRISPR/Cas is originally linked to the adaptative immune system present in prokaryotes to specifically target viruses. These systems may be used as gene editing tools and applied for the prevention and control of plant viruses in the field [4,7]. The first CRISPR/Cas systems studied were very useful for DNA targeting, however, more recently, the CRISPR/Cas13 was identified, which can specifically cleave single-stranded RNA (ssRNA) in eukaryotes. This has placed CRISPR/Cas as a promising tool for the development of immunity against a wide range of RNA viruses, which are the most abundant class of viruses in plants. In addition, among the existing DNA plant viruses, many contain an RNA intermediate [8].

The present chapter aims to bring together the latest information on CRISPR/Cas systems, including their origin, components and classification, and diverse applications, namely, to control plant viruses. Considering the unique characteristics of CRISPR/Cas13 systems, such as their robustness, preciseness, and versatility, this review will focus on CRISPR/Cas13 systems to control plant viruses. We also discuss the limitations and future challenges of CRISPR/Cas13 in the development of virus-resistant plants for future precision breeding and sustainable agriculture.

16.2 OVERVIEW OF CRISPR/CAS TECHNOLOGY

16.2.1 Origin of CRISPR/Cas Technology

For many years scientific efforts have been made to find new technologies able to modify eukaryotic genomes [9]. New solutions are often found in prokaryotes, which provide innovative and nature-based solutions for gene editing, such as reporter genes (lacZ) [10], strong inducible gene expression (tetracycline system) [11], and effective conditional mutagenesis (cre/loxP system) [12].

CRISPR/Cas is one of the most modern examples of genetic engineering tools that were found and developed from prokaryotes. Over the past decades, key findings and progress in prokaryote research allowed the launch of this technique [9]. Atsuo Nakata and his research team, in 1987, first reported DNA repeats with dyad symmetry, in Gram-negative bacteria *Escherichia coli* K12, which would become known as CRISPR [13]. The first studies with insights on CRISPR functionality were reported in archaea and published in 1993 and 1995 [14,15]. In 2006, CRISPR/Cas systems were suggested as bacterial defense mechanisms, due to the discovery of spacer sequences, which were homologous to DNA sequences from bacteriophages or plasmids [16]. One year later, this technology was experimentally demonstrated to take part in acquired immunity against bacteriophages, since it was confirmed that by acquiring spacers that match the viral genome, a sensitive bacterial strain can develop resistance to infection [17]. The following studies on CRISPR/Cas systems allowed comprehensive insights into its structure, components, and functions, leading to reports demonstrating functional CRISPR/Cas systems as competent genome editing tools in 2012 and 2013 [18,19].

Since 2013, when it was first applied in plants, CRISPR has been used for genome editing in a wide range of crops, many of which have high-value agricultural traits [20,21]. The most recent CRISPR/Cas technologies are particularly important because they can change nucleotides precisely, which can have major impacts on agriculture. In addition, this technology can go beyond editing specific *loci* for crop improvement, being capable of promoting gene regulation and protein engineering. Therefore, CRISPR/Cas technologies have already shown high potential for fundamental biological research and have raised the prospect for multiple new applications [22].

16.2.2 Components and Classification of CRISPR/Cas Systems

The CRISPR/Cas units, adaptative immune systems present in archaea and bacteria, offer protection against foreign DNA or, sometimes, foreign RNA by specifically recognizing sequences of the invader. CRISPR/Cas *loci* consist of a CRISPR array, which is composed of short direct repeats separated by spacers (short variable DNA sequences), bordered by different *cas* genes [23] (Figure 16.1).

CRISPR/Cas immunity comprises three basic steps: adaptation, expression, and interference (Figure 16.1). These systems work by memorizing phage infections, and the first step is adaptation or acquisition, which consists of the incorporation of protospacers, which are fragments of foreign DNA from invading organisms into the CRISPR array. After incorporation, the spacers allow a specific defense against following invasions [23]. Protospacer acquisition and insertion into the CRISPR array is mediated by Cas1 and Cas2, a complex of Cas proteins that together with additional proteins, regulate this process, being able to measure and cut out a piece of exactly the right size to insert a new spacer. All spacers are always flanked by repeats on each side, as the system incorporates a new repeat in the process [24].

CRISPR/Cas systems store these spacers in the DNA as a way of remembering the infection but do not use the DNA to directly recognize subsequent infections. Instead, the CRISPR array is transcribed as a precursor transcript (pre-crRNA) which is processed and matured into CRISPR RNAs (crRNAs). These crRNAs are used to find new invading viruses and can be degraded and recycled without destroying the original memory [23]. Pre-crRNAs can be bound to a single multidomain protein or a multisubunit effector complex. An endonuclease subunit of the multisubunit effector complex or an alternative mechanism involving bacterial RNase III and other RNA species called transactivating CRISPR RNA (tracrRNA), are responsible for the processing and maturation of the pre-crRNA into crRNAs [25].

The last phase in CRISPR immunity, interference, allows the cleavage of the invading phage's DNA upon infection. During this stage, mature crRNAs, aided by Cas proteins, the so-called effector complex or surveillance complex, recognize and cleave the cognate DNA or RNA. Once the phage's DNA or RNA is cleaved and its replication is incapacitated, the infection is over [23].

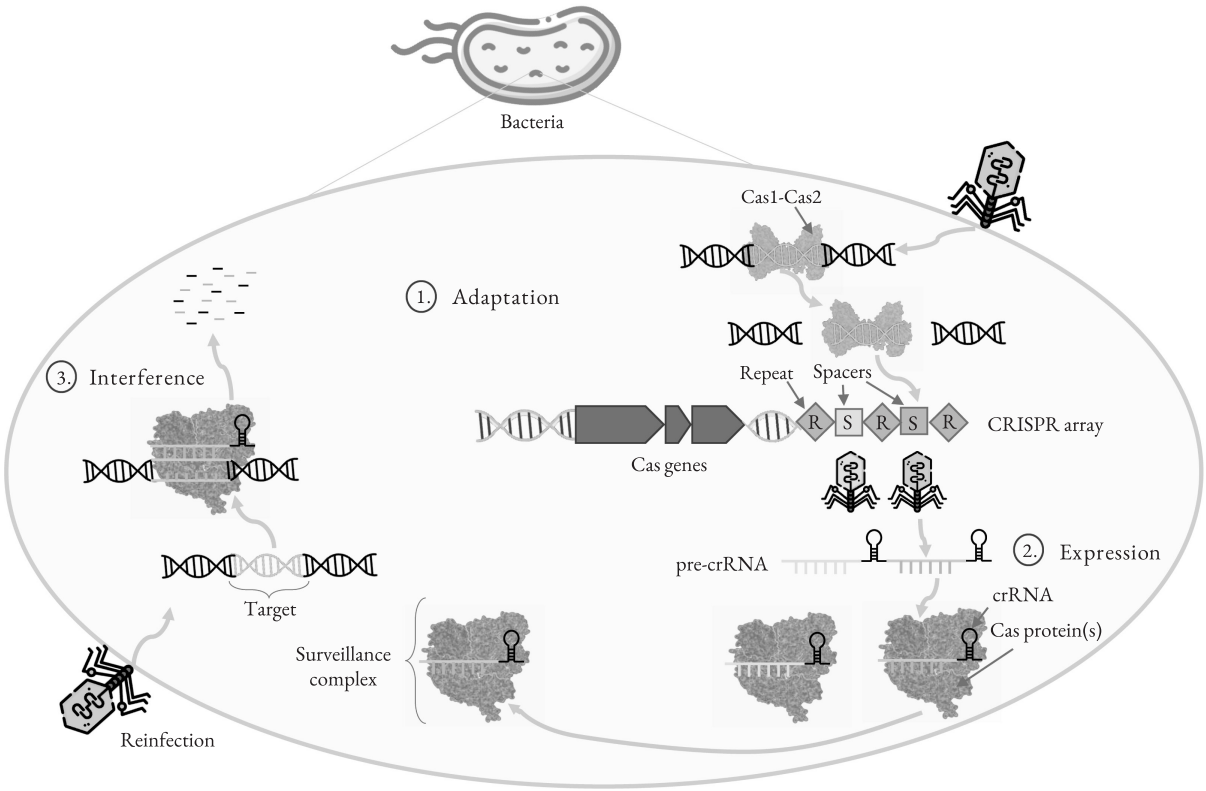

FIGURE 16.1 CRISPR/Cas systems components and immunity steps.

As a result of their evolutionary arms race with pathogens, the Cas protein sequences and the genomic architecture of CRISPR/Cas *loci* display a remarkable diversity typical of antiviral defense mechanisms [23,26,27]. However, this variability poses a major challenge for achieving a coherent annotation and a rather simple classification of CRISPR/Cas systems, which would clarify their origins and evolution and keep track of new variants. Nonetheless, for further progress in CRISPR research, a coherent classification scheme is essential [28,29].

CRISPR/Cas systems classification approaches have created and adopted a combined, semi-formal method, based on signature genes and distinctive gene architectures, allowing the assignment of these systems to types and subtypes. Therefore, signature genes are specific for each type and subtype of CRISPR/Cas systems. In addition, sequence similarity between multiple Cas proteins, the phylogeny of Cas1 (the best conserved Cas protein), the organization of the *loci* and the structure of the CRISPR themselves is of major importance for this classification [23,28,29].

As mentioned above, the classification of CRISPR/Cas systems is complicated because of their diversity and constant evolution. Not even Cas1 can be considered as a universal Cas protein since it fails to adequately represent the relationships between all CRISPR/Cas systems and cannot be used as a phylogenetic marker. Therefore, the application of the multiple criteria previously mentioned results in the classification scheme presently used for CRISPR/Cas systems, which separates them into two different classes, according to the design principles of the effector complex. These complexes can have several Cas proteins, having a multi-subunit design, which is the case of Class 1 systems. On the other hand, Class 2 systems have a single, large multidomain protein [23,28] (Figure 16.2).

Class 1 systems are classified into three types – I, III, and IV. Types II, V, and VI belong to Class 2 CRISPR/Cas systems. In each type, the design of the effector complex gene is different, having unique signature proteins, which allows its classification into several subtypes, encompassing subtler differences in *locus* organization and encoding subtype-specific Cas proteins [23,26,29,30] (Figure 16.2).

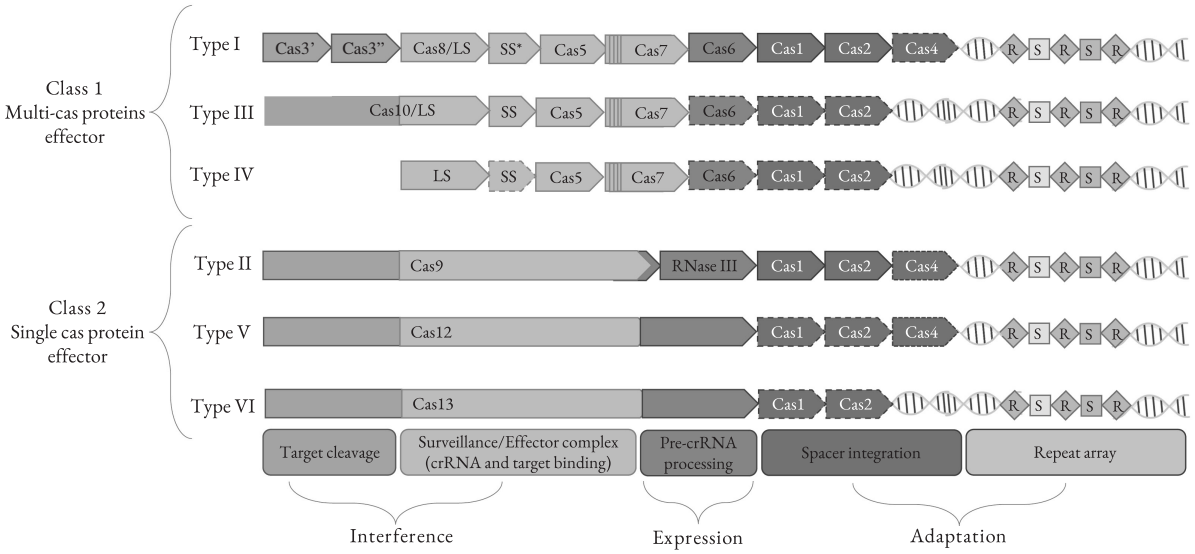

FIGURE 16.2 Class 1 and Class 2 CRISPR/Cas systems: key functions and gene organization. Genes are shown as blocks and the same color represents genes with a homologous function. Dashed outlines indicate unessential genes. An asterisk indicates the putative small subunit (SS) that is thought to be attached to the large subunit in many type I subtypes.

Accordingly, one of the main aspects that differentiate Class 1 from Class 2 systems is the pre-crRNA processing. In Class 1 systems, a complex of several Cas proteins – Cascade (CRISPR-associated complex for antiviral defense) – catalyzes the maturation of pre-crRNAs to crRNAs. The Cascade complex is responsible for binding the pre-crRNA and recruiting a supplementary Cas protein (nuclease), directly responsible for the processing (usually Cas 6, but it can also be Cas5) [31–33].

In Class 2 systems, for type II, considered as a prototype, an RNAse III enzyme catalyzes the processing of pre-crRNAs, aided by additional RNA species, the tracrRNA. On the other hand, in types V and VI, different nuclease activity, belonging to the same large effector protein, is observed, but this process is not completely understood. However, for subtype, V-B systems tracrRNAs have been identified, though RNAse III is not present, and the cleavage enzyme is still unknown [25,34,35]. DNA editing is possible using types II and V Cas proteins, whereas RNA editing can be done by applying type VI Cas proteins [4].

The interference stage is also different for each class of CRISPR/Cas systems. In Class 1 systems, the effector complex (crRNA and Cas proteins) recognizes the protospacer sequence in the target and recruits Cas3. The helicase domain of Cas3 unwinds the target dsDNA, and the nuclease domain cleaves the foreign DNA. In type III systems, the nuclease involved is a part of the effector complex. Hence, no helicase is involved but DNA cleavage first requires cleavage of RNA transcripts by a distinct CRISPR-associated RNase [36,37]. In Class 2 systems, cleavage is accomplished by the nuclease domain(s) of the large effector protein [25,35].

16.2.3 CRISPR/Cas Systems for Crop Improvement

CRISPR/Cas systems are highly specific and robust, allowing precise genome editing which can introduce beneficial traits to enhance agricultural sustainability. Therefore, this technology has altered plant molecular biology exceeding expectations. Furthermore, the variety of emerging technologies based on CRISPR/Cas systems has expanded the range of fundamental research and plant biology [22].

Genome editing using CRISPR/Cas has benefits compared to the basic strategies that use sequence-specific nucleases (meganucleases [38], zinc-finger nucleases [39], or transcription activator-like nucleases [40]) to induce DNA double-strand break (DSB) at a target site and homology-directed repair (HDR). These basic strategies have shown to be effective for plant genome editing, however, their creation requires complex protein engineering. On the other hand, CRISPR/Cas systems can be easily engineered to introduce DSBs at any chosen target location with reduced costs [41].

Precise genome editing in plants based on CRISPR/Cas systems, such as deaminase-mediated base editing or reverse transcriptase-mediated prime editing technologies, are alternative genome editing technologies in which DSB is not involved and a donor DNA is not needed, being more efficient than HDR in plants [22]. In addition, new technologies based on CRISPR/Cas9, such as cytosine base editor (CBE) [42], adenine base editor (ABE) [43], dual base editing [44], and CBE-based precise DNA deletion [45], were also developed for precise genome editing in plants.

CRISPR/Cas technologies can induce precise nucleotide changes, which can have a high impact on agriculture. Nevertheless, CRISPR/Cas potential goes much further than simply editing specific *loci* for crop improvement, as the development of new plant biotechnologies based on these systems has shown the capacity for gene regulation and protein engineering [22].

Regarding CRISPR/Cas upgrade applications for crop improvement, a great number of studies, mostly using CRISPR/Cas9, have demonstrated the successful improvement of several crop characteristics such as yield [46–48], quality [49–51], disease resistance [52,53] and herbicide resistance [54,55]. In addition, many applications in breeding technologies have emerged, targeting reproduction-related genes using CRISPR/Cas systems, such as haploid induction, generating male sterile lines, fixation of hybrid vigor, and manipulating self-incompatibility [22].

Concerning disease resistance, the role of CRISPR/Cas systems against plant viruses is highlighted in the present chapter. Over the past few years, researchers have been able to use CRISPR/Cas system-mediated gene editing to create resistance against pathogens, specifically against viruses, which are known to infect many economically important crops, being a great threat to food security worldwide. The first studies on CRISPR/Cas systems against plant viruses used CRISPR/Cas9 that targeted DNA viruses, but then the later use of CRISPR/Cas13 allowed to target RNA viruses. Generally, there are two main strategies to control plant viruses using CRISPR/Cas technologies: (1) targeting viral genome (DNA or RNA) to inhibit replication and infection or (2) manipulating host susceptibility factors essential for viral infection [4,56]

Targeting of the viral genome to protect plants against viruses using CRISPR/Cas technology was first studied and designed to target DNA viruses. Upon entry of a DNA virus into the plant cell, the single guide RNA (sgRNA) fused to crRNA and tracrRNA from CRISPR/Cas9, in this case, is complementary to a sequence from the DNA target. The Cas9/sgRNA first binds and then cleaves the DNA target [56]. Specifically, the CRISPR/Cas9-mediated editing tool was successfully used as a defense mechanism against DNA plant viruses such as members of the family *Geminiviridae* [57–59] and *Cauliflower mosaic virus* [60]. These studies were mostly done in model plants, which is the case of *Nicotiana benthamiana* and *Arabidopsis thaliana*, but more recent studies have attempted CRISPR/Cas-mediated resistance in crops. Accordingly, a CRISPR/Cas9 machinery was engineered in tomato plants to target *Yellow leaf curl virus* [61] and in barley against *Wheat dwarf virus* [62].

Nevertheless, RNA strands make up most plant viruses' genomes and even DNA plant viruses exhibit an RNA intermediate at some point in their life cycle, highlighting the importance of effectors with RNA specificity as systems of choice to target viral genomes and protect plants. For RNA viruses or RNA intermediates of pathogens with DNA genomes, studies were developed with both Cas9 and Cas13 proteins guided by a sgRNA or a crRNA, respectively. These systems have proven to be successful in targeting RNA viruses, being able to cleave their genome, and preventing further infection [56]. The first description of CRISPR/Cas-based plant immunity against an RNA virus targeted *Cucumber mosaic virus* and *Tomato mosaic virus* using FnCas9 variant. Reduced

virus accumulation was observed in transgenic tobacco plants, as well as in *Arabidopsis* plants [63]. Applications of RNA virus interference by CRISPR/Cas13 in plants have also been described in recent literature, showing promising results. For instance, LshCas13a successfully interfered against *Turnip mosaic virus* in both *N. bentamiana* and *A. thaliana* [64]. In addition, CRISPR/Cas13 systems allow the targeting and degradation of viral RNA genomes, conferring resistance to an RNA virus in monocot plants. Namely, this was observed against *Rice black-streaked dwarf virus*, *Rice stripe mosaic virus,* in transgenic rice plants harboring the CRISPR/Cas13a system [65]; against multiple *Potato virus Y* strains in transgenic potato plants [66]; and against *Grapevine leafroll-associated virus 3* in grapevine [67]. These reports are examples of a great number of studies that are available and being developed worldwide on this revolutionizing technology for direct targeting of RNA viruses.

Moreover, CRISPR/Cas system-mediated resistance to plant viruses can also target host factors. This can be done by gene knockout of the susceptibility (S) genes, editing of the promoter regions, insertion of resistance genes by HDR, or mimicking polymorphisms by targeted nucleotide modification [4,56].

The selection of desirable traits commonly leads to a loss of genetic diversity and increased vulnerability to biotic and abiotic stresses [68]. Therefore, some studies point out the domestication of wild species or the use of semi-domesticated crops as an appealing way to help meet the continuously growing demand for food and nutrition, a consequence of a growing world population. Since the traditional process of wild species domestication is lengthy, because it involves many *loci* but just a few of them have key roles for the desired outcome, CRISPR/Cas is the perfect technology to accelerate the process, having the ability for precise genome editing [69]. Several pioneering and foundation studies on ways to accelerate this process have already been conducted, namely on *Solanum pimpinellifolium*, a putative ancestor of tomato [70,71], and *Oryza glaberrima*, the African rice [72]. Nevertheless, this process still includes several bottlenecks, and further studies are necessary to provide basic knowledge on the genetics of wild species and domestication genes.

Concerning plant biotechnology employing CRISPR/Cas systems, many studies have been carried out, specifically on CRISPR/Cas delivery in plants, gene regulation, multiplex genome editing, mutagenesis, and directed evolution. The application of this technology in plants requires a robust and universal delivery system. Biolistic bombardment and *Agrobacterium*-mediated delivery have been used for decades, but have some limitations. Biolistic bombardment can deliver genetic material beyond the rigid cell walls, using mechanical force however, efficiency is not very high and genome sequences can be damaged. In *Agrobacterium*-mediated delivery, although the integration of foreign DNA is inevitable, *Agrobacterium* can efficiently infect a large range of plants. Moreover, both methods require lengthy tissue culture procedures. *De novo* meristem induction, virus-assisted gene editing, and gene editing with haploid inducers, are delivery systems developed to undermine the limitations of traditional delivery systems. These new tools allow genome manipulation with no need for exogenous DNA, which has advantages over traditional breeding since target mutations are reduced and public concerns toward transgenic lines cease to be a problem [22].

16.3 CRISPR/Cas13 SYSTEMS

16.3.1 Discovery, Classification, and Structure of CRISPR/Cas13 Systems

CRISPR/Cas13 systems belong to Class 2 type V systems, with one multifunctional Cas13 effector protein, containing two higher eukaryotes and prokaryotes nucleotide-binding domains (HEPN) responsible for RNase activity. Similar to the previously mentioned for CRISPR/Cas9, the Cas13 associated with crRNA forms the effector complex, which in this case is an RNA-guided complex that targets and cleaves ssRNA. The nuclease domain(s) of the large effector protein is responsible for processing the pre-crRNA processing and cleavage of the ssRNA [8,25,35].

Applying data analysis and bioinformatics approaches, Shmakov and coworkers analyzed the whole microbial genome sequences from the National Centre for Biotechnology Information, based on the incidence of Cas1 (most conserved Cas protein gene) aiming to identify the unclassified candidate Class 2 CRISPR *loci*. As a result, a new Class 2 effector type was predicted, the C2c2 (Class 2 candidate2) or VI-A, using Cas1 as the seed [34]. This first presumed type VI effector, C2c2 or VI-A, demonstrated unique properties compared to any other Cas protein, therefore, it is now designated Cas13a and was assigned to a novel type (Class 2, type VI) [30,56,73]. The hypothesis that Cas13a presents an association between HEPN domains and RNase, acting as an RNA-guided RNase and being able to target RNA, was experimentally confirmed when Abudayayyeh and coworkers (2016), showed that type VI Cas13a effector possessed a ssRNA-targeting capability in RNA bacteriophage MS2 [74], facilitating interference and pre-crRNA processing [75].

Therefore, Cas13a was the first type VI ribonuclease identified (with an average size of 1250 amino acids) that can efficiently target and degrade ssRNA, but not double-stranded RNA (dsRNA). This system was characterized in *Leptotrichia sharii* (Lsh) [8,76], although it has many orthologs such as *Listeria seeligeri* (Lse), *Leptotrichia wadei* (Lwa) [77], *Leptotrichia buccalis* (Lbu) [75] and *Lachnospiraceae bacterium* (Lba) [78]. The Cas13a *locus* is composed of an adaptation module (Cas1 and Cas2), two HEPN domains, and a CRISPR array [73,74] (Figure 16.3). Thus, the crRNA-Cas13a complex is bilobed consisting of a nuclease lobe (NUC lobe) and a crRNA recognition lobe (REC lobe) [73]. NUC lobe contains HEPN domains, HEPN1 and HEPN2, with a linker domain in between, located on the outer surface and responsible for the cleavage of the target RNA outside the binding region. However, when this happens, the catalytic site of HEPN is exposed and available to all RNAs in a solution, which might result in some unspecific cleavage [56,79]. HEPN1 domain has the HEPN1 I subdomain and the HEPN1 II subdomain with a Helical-2 domain in between [79]. The REC lobe consists of an N-terminal domain (NTD) and a Helical-1 domain that catalyzes the maturation of the crRNA [79]. In these systems, the CRISPR array generally

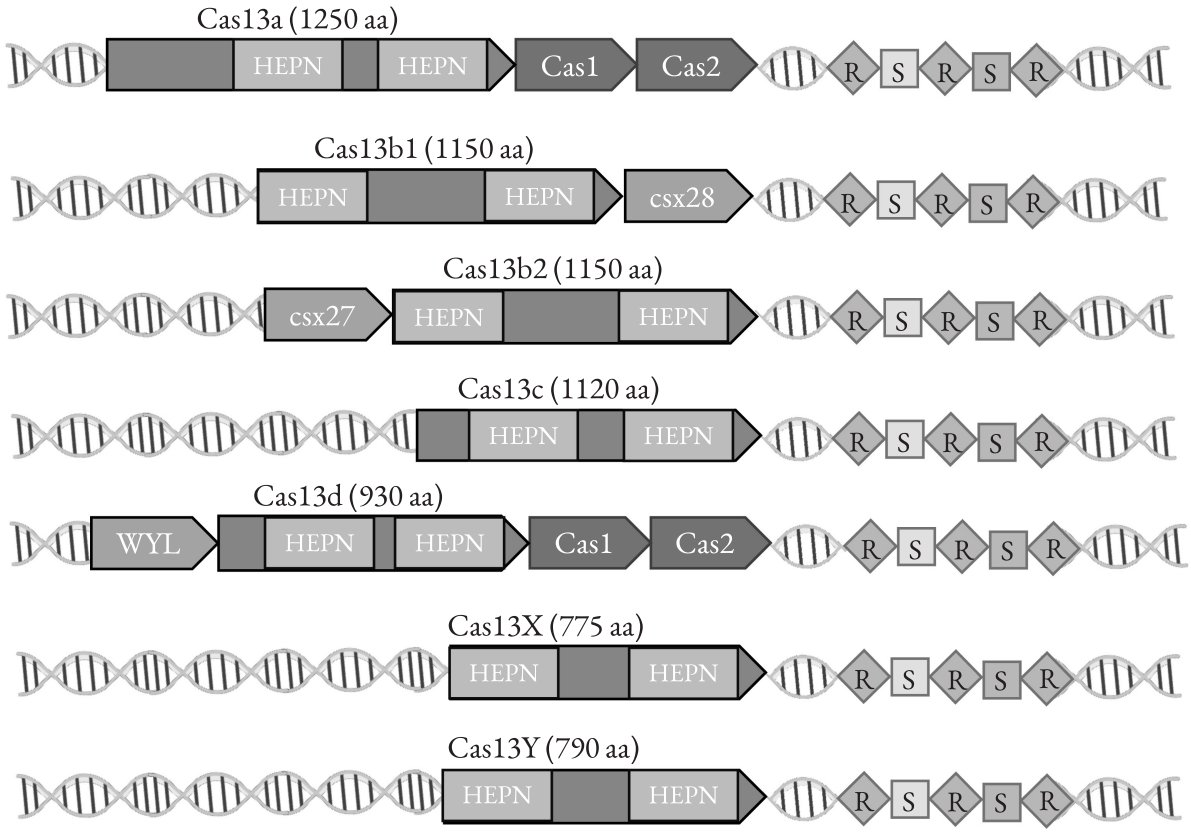

FIGURE 16.3 Constitutions of the different CRISPR-Cas13 subtypes. Genes are shown as arrows.

comprises a 5′ 28 nucleotides (nt) direct repeat (DR), which is typical of each ortholog, and a 28–30 nt spacer sequence (complementary to the RNA target). Some orthologs, such as LshCas13a, have a single base protospacer flanking site (PFS) preference, comprising A, U, or C nt bases (non-G) at the 3′ end of the spacer sequence (guide sequence) [8,74], whereas LwaCas13a and LbuCas13a do not show PFS preference. LwaCas13a system did not require a PFS motif, which improves the flexibility of Cas13a and makes it the variant of choice [77,80].

Nevertheless, not all CRISPR/Cas systems are autonomous, because Cas1 protein may be absent. In this case, they depend on adaptation modules (Cas1 and Cas2) of other CRISPR/Cas systems of the genome. Accordingly, the detection of these nonautonomous systems in the previous analysis, based on Cas1, was not possible [23,34,73]. However, following studies investigating the CRISPR/Cas *loci*, using the CRISPR repeat arrays as seed, were able to identify new Class 2 subtypes lacking the adaptation module. This research allowed the identification of two additional Class 2 type VI effector subtypes, containing HEPN domains: VI-B or C2c6 (Cas13b) and VI-C or C2c7 (Cas13c) [29,30,81,82].

The different subtypes that exist in type VI systems emphasize the diverse locations of HEPN domains of Cas13 and other additional features of the *locus* architecture. Also, the sequence similarity of the catalytic motif of the HEPN domain between the three groups (VI-A, VI-B, and VI-C) is extremely low which justifies their separation. Regarding subtype VI-B, although it also presents two HEPN domains and targets ssRNA, it encodes additional proteins that contain predicted transmembrane domains, which are significantly different from VI-A subtype, Csx27 and Csx28 (Figure 16.3). Csx27 can repress RNA targeting and Csx28 can enhance RNA cleavage. According to phylogenetic analysis, VI-B systems suffered an evolutionary divergence that resulted in VI-B1 and VI-B2 variants with distinct architectures of these associated predicted membrane proteins [56,73,82].

Cas13b was discovered using bioinformatic methods in species of Gram-negative bacteria, namely *Porphyromonas* sp. (PguCas13b) and *Prevotella* sp. (PspCas13b). However, there are other identified Cas13b orthologs such as *Bergeyella zoohelcum* (BzCas13b). These systems have the two HEPN domains positioned at N and C protein terminals and devoid of Cas1 and Cas2 proteins but include, as mentioned before, two additional proteins, Csx27 and Csx28 [82]. Cas13b has an average of 1150 amino acids and its DR on the 3′ end of crRNA (spacer length 30 nt) is in contrast to the 5′ DR present in Cas13a, and needs a double-sided PFS. BzCas13b and PguCas13b prefer 5′ PFS of A, U, or G (non-C) and 3′ PFS of NAN or NNA, maximizing their targeting ability. PspCas13b is an exception because it has no PFS requirement [8,80]. RNA-targeting in eukaryotes using PspCas13b has shown to have constantly greater efficiency than LwaCas13a, not only because it lacks the need for PFS, but also because it has demonstrated a lack of RNA collateral damage. Accordingly, PspCas13b is preferred for targeted RNA cleavage [73,83].

Cas13c was also first identified using a bioinformatic approach in *Fusobacterium* and *Clostirdium* but its functional characterization is much less complete than other Cas13 types. This protein has a similar *locus* and structure of the crRNA as Cas13a, with 1120 amino acids of length, however, the adaptation module (Cas1 and Cas2) is also absent, similar to Cas13b (Figure 16.3). This protein presents a DR on the 5′ end of crRNA with a spacer size of 28–30 nt. There is a lack of research available on this type of Cas13, mostly because it is less efficient at RNA targeting and interference in comparison to other Cas13 subtypes. The few studies that exist mostly employ the Cas13c ortholog *Fusobacterium perfoetens* (FpeCas13c) [8,73,80].

After upgrades on bioinformatic processes and with access to a higher number of datasets on genomics and metagenomics, it was possible to reveal a new Class 2 type VI effector protein, the Cas13d, subtype VI-D. This effector protein was identified mainly in *Eubacterium* and *Ruminococcus* [73,84]. The Cas13d from *Ruminococcus flavefaciens* XPD3002 (CasRx/RfxCas13d) is one of the most characterized variants [8]. Cas13d is smaller than Cas13a, Cas13b, and Cas13c effector proteins, with about 930 amino acids. REC lobe has NTD and Helical-1 domains and NUC lobe has domains HEPN-1, HEPN-2, and Helical-2. In addition, this system comprises a WYL domain with accessory proteins, one of which might positively modulate RNase activity, either targeted or

collateral (Figure 16.3). The crRNA of Cas13d has a 30 nt 5′ DR followed by a variable spacer that can range from 14 to 26 nt. This CRISPR/Cas13 subtype is known for its versatility, since it has no PFS constraints, and employs rigorous sequence-specific RNA cleavage, which is promising for enhanced RNA interference compared to other systems [8,73,80].

Recently, other Cas13 protein variants were identified, Cas13X and Cas13Y, having the smallest size of 775 and 790 amino acids, respectively. Little is known about the structure of these proteins, they have two HEPN domains, located in the N- and C-terminus of the proteins, similar to the Cas13b subtype (Figure 16.3). Among Cas13X and Cas13Y, Cas13X1 exhibited the highest knockdown efficiency and showed no PFS bias [80,85].

BOX 1- CRISPR/CAS13 SYSTEMS IN A NUTSHELL

- Cas13 protein is the signature gene for type VI CRISPR systems. Based on the phylogeny of Cas13, features, and functional characterization, type VI CRISPR systems are classified into six subtypes: VI-A (effector protein is Cas13a/C2c2), VI-B (effector proteins are Cas13b1/C2c6 and Cas13b2), VI-C (effector protein is Cas13c/C2c7), VI-D (effector protein is Cas13d), VI-X (effector protein is Cas13X) and VI-Y (effector protein is Cas13Y) [8,29,30,73,74].
- The identification of these subtypes was first accomplished by using data mining and bioinformatic approaches, either by using Cas 1 as a seed (for the identification of Cas13a) or by using the CRISPR array as a seed (for the following Cas13 subtypes) [23,29,34,73].
- Although the effector protein sizes and primary sequence differ among Cas13 subtypes, they all share a common feature, which is the presence of two HEPN domains that provide RNase activity. An RNA-guided RNA targeting complex is formed with a crRNA to recognize and cleave ssRNA targets [8,30,34,73]. Therefore, all Cas13 proteins have two enzymatically distinct RNase activities, including processing pre-crRNA and degradation of target RNA [8,30,75].
- Cas13 effectors have a bilobed structure with NUC and REC lobes however, the sequence of nucleotide bases and the organization of the domains can be very different [79]. The NUC lobe contains HEPN domains, for RNA cleavage, which have different locations and are uniquely spaced based on the subtype of Cas13 protein [30,34,73]. In Cas13a, Cas13c, and Cas13d the HEPN domains are located at the centre and C-terminus. On the other hand, in Cas13b, Cas13X, and Cas13Y, HEPN domains are present at the N-terminus and C-terminus of the proteins. These domains can cleave the target RNA but also show nonspecific collateral cleavage that results in the degradation of the RNA near the Cas13 system. Nonetheless, some CRISPR/Cas13 variants showed a lack of collateral RNA damage, such as PspCas13b [73,83]. REC lobe has an NTD and a Helical 1 domain functional for pre-processing and interaction with sgRNA [79]. The length of the crRNA sequence varies from 24 to 30 nt.

Table 16.1 shows a schematic summary of CRISPR/Cas13 classification, according to the effector protein and structural composition.

16.3.2 Molecular Mechanism and Application of CRISPR/Cas13 Systems against Plant Viruses

As well as in other CRISPR/Cas systems the molecular mechanisms of adaptative immunity of CRISPR/Cas13 encompasses three steps: adaptation, expression, and interference. The adaptation

TABLE 16.1
CRISPR/Cas13 Classification

Type of Cas13	Length (aa)	Orthologs	Structural Composition	Functional Region	Reference
Cas13a or C2c2 (VI-A)	1,250	LshCas13a LseCas13a LwaCas13a LbuCas13a LbaCas13a	HEPN domains at center and C-terminus; 5′ end DR; 3′ non-G PFS preference (Lwa and LbuCas13a with no preference).	SsRNA (28–30nt spacer sequence)	[74,75]
Cas13b or C2c6 (VI-B)	1,150	PguCas13b PspCas13b BzCas13b	HEPN domains at N and C-terminus; 5′ non-C PFS preference; 3′ PFS NAN/ NNA (except PspCas13b); 3′ end DR.	ssRNA (30nt spacer sequence)	[82,86]
Cas13c (VI-C)	1,120	FpeCas13c	HEPN domains at centre and C-terminus; 5′ end DR; No PFS preference.	ssRNA (28–30nt spacer sequence)	[30,87]
Cas13d (VI-D)	930	RfxCas13d	HEPN domains at centre and C-terminus; 5′ end DR; No PFS preference.	ssRNA (23–30nt spacer sequence)	[80,88]
Cas13X (VI-X)	775		HEPN domains at N and C-terminus; No PFS preference.	ssRNA	[80]
Cas13Y (VI-Y)	790		HEPN domains at N and C-terminus; No PFS preference.	ssRNA	[80]

Source: Adapted from Kavuri et al. [8].

step consists of acquiring new spacers; however, this mechanism is poorly understood in type VI systems compared to others. This is mainly because some CRISPR/Cas13 systems subtypes lack Cas1 and Cas2, the known adaptation modules, so they might require adaptation factors of other systems existing in the genome [34,89]. In the expression phase, the production of the crRNA and the effector nuclease takes place (surveillance complex). Transcription of the pre-crRNA into crRNA is accomplished by the REC lobe of Cas13, in contrast to other CRISPR/Cas systems that require either a tracrRNA or an endogenous RNase [75,89]. A hairpin flanked by a spacer sequence is formed by the DR region and the spacer within a crRNA is set for a precise RNA target. This complex will mediate recognition and binding of the CRISPR/Cas13 to the target RNA which will be surrounded by the nuclease core, and the catalytic nuclease is activated to efficiently cleave the target RNA [73,89]. Unlike, for instance, Cas9 effectors, Cas13 proteins do not need the existence of a protospacer adjacent motif (PAM) to recognize target RNA; however, some Cas13 subtypes show a preference for specific nucleotides flanking the 3′ region of the protospacer, the PFS [77]. In addition, collateral cleavage can also happen upon target recognition, which means not only do they target RNA but also can cleave indiscriminate bystander RNA. This is because the catalytic region and the crRNA: target RNA binding site are located on opposite sides, which makes it reachable from the outside of the complex. Nevertheless, this collateral cleavage activity of Cas13 is still controversial in eukaryotes, since it seems to be absent in some organisms [90].

The first CRISPR/Cas13 system to be studied for RNA-targeting used the effector protein ortholog LshCas13a. Immunity against *Turnip mosaic virus* (TuMV) in *N. benthamiana* and *A. thaliana* was developed and demonstrated that this system mediates specific RNA virus targeting in plants, although with moderate efficiency. LshCas13a interference was tested by transforming a plant codon-optimized LshCas13a into *N. benthamiana* leaves for transient and transgenic expression, using four different crRNAs (GFP1, GFP2, the *helper component proteinase silencing suppressor,* and *coat protein* sequences) to target the GFP-tagged TuMV genome. The observation of the GFP signal under UV light allowed the measurement of viral incidence. Collected data showed

a 50% reduction in the transcript levels for GFP2 and the *helper component proteinase silencing suppressor.* Another similar study in *A. thaliana* proved the heritable immunity against TuMV up to T2 generation [64,91]. In addition, LshCas13a has become a promising Cas13 ortholog to accomplish immunity against a wide range of RNA viruses in different plant species, including against *Potato virus Y* in *Solanum tuberosum* [66] and against *Southern rice black-streaked dwarf virus* and *Rice stripe mosaic virus* in either *N. benthamiana* and *Oryza sativa* [65].

In addition to the well-characterized LshCas13a, other protein effectors have been studied to identify better variants against plant viruses, that would allow higher interference efficiency. For instance, LwaCas13a has been reported to mediate a stronger RNA-targeting activity than LshCas13a however, it does require a stabilizer fusion, such as msfGFP for an efficient interference activity [77]. In addition, other Cas13 subtypes were also exploited for developing resistance to plant viruses, such as BzCas13b, PspCas13b, and RfxCas13d [92].

Studies have shown that LshCas13a, LwaCas13a, BzCas13b, PspCas13b and RfxCas13d can be used against TuMV, TMV and *Potato virus X* in *N. benthamiana* [8,92] and LshCas13a, LwaCas13a, PspCas13b and RfxCas13d can also be efficient against *Cucumber mosaic virus* and *Sweet potato chlorotic stunt virus-RNase3* in *N. benthamiana* and *Ipomoea batatas* [8,93]. The virus RNA interference data suggested that all other variants (LwaCas13a, PspCas13b and RfxCas13d) were more efficient than LshCas13a [92]. Furthermore, RfxCas13d was identified as the most effective Cas13 ortholog for RNA targeting in *N. benthamiana* in transient and stable assays and showed to be a highly specific RNA targeting system that lacks collateral activities *in planta*. Multiplexed virus interference was also achieved using this ortholog, by targeting two different RNA viruses simultaneously [92].

16.3.3 Potential Limitations and Future Directions of CRISPR/Cas13 Systems

Despite all of the biotechnical and agricultural applications of CRISPR/Cas13 systems, there are still limitations regarding this technology. Despite the many studies that have arisen, it is a very recent technology that needs further investigation in different plant species and against diverse plant viruses. The newly identified subtypes of CRISPR/Cas13 systems (Cas13X and Cas13Y) are not fully characterized and their mechanism is not completely understood [8,73]. Therefore, additional information is needed on these effector proteins to comprehend their potential for RNA targeting and generating immunity against plant viruses.

In addition, the collateral RNase activity present in these systems may lead to the degradation of non-target RNAs, which was observed *in vitro* and bacterial cells [90]. However, in plant cells, no collateral activity was observed and even though there are Cas13 variants that showed to lack this collateral activity, it is important to ensure that this will not be a problem in future studies on CRISPR/Cas13 immunity in plants against RNA viruses [73].

Another important concern would be that these systems rely on a spacer in crRNA that is specific for a target ssRNA, so cleavage sites and cleavage patterns for a precise target transcript cannot be changed, otherwise, the system would not be successful for RNA interference [74,82]. Given the rapid adaptability of plant viruses, mutations in their genome can occur or new viruses may emerge, which will demand adjusting these systems to ensure plant immunity.

CRISPR/Cas13 RNA targeting systems are very promising RNA technologies with multiple advantages over the ones that previously existed, such as their robustness, specificity, easy design, and affordable price. Cas13 structural and functional variants that have a single effector protein allow not only studies on viral RNA interference, which have been the main focus but also Cas13-mediated knockdown of endogenous mRNA and targeting of non-coding RNAs (long non-coding RNA, microRNA, and circular RNA) to understand their role in plants [8,73].

16.4 CONCLUSION

Plant viruses can cause destructive diseases in several agricultural systems which highlights the need for new methods of control and monitoring, in view of a more sustainable agriculture, able to prevent extreme economic impacts worldwide and guaranteeing both food safety and food security. The development of virus-resistant plants is of utmost importance nowadays, since plant viruses can easily spread throughout crops and there are no chemical products available for their control in the field, making prevention essential to avoid catastrophic losses.

CRISPR/Cas systems have been studied over the last decades. These are adaptative immune systems that prokaryotes present as a result of their defense against viruses, and that have been found by scientists to have potential as gene editing tools. CRISPR/Cas immunity involves adaptation, expression, and interference stages and a variety of different systems has already been identified. According to multiple criteria, such as signature genes, the phylogeny of Cas1 (the best conserved Cas protein), the organization of the *loci,* and the structure of the CRISPR themselves, CRISPR/Cas systems are classified within Class 1 and Class 2, with different types and subtypes. Several applications for this technology have been found and some are still being studied, either for crop improvement (yield, quality, disease resistance, herbicide resistance, breeding technologies, or domestication of wild species) or plant biotechnology (delivery systems, gene regulation, multiplex editing and mutagenesis and directed evolution).

In this chapter, we focus on the use of CRISPR/Cas systems against plant viruses. The role of CRISPR/Cas13 was highlighted, mostly because of recently described systems that can target and cleave RNA virus genomes or transcripts, which is extremely important since most plant viruses are RNA viruses or have RNA intermediates. Cas13 is the signature gene for CRISPR/Cas13, type VI CRISPR systems, which can be classified into six subtypes (VI-A, effector protein Cas13a; VI-B, effector protein Cas13b; VI-C, effector protein Cas13c; VI-D, effector protein Cas13d; VI-X, effector protein Cas13X; and VI-Y, effector protein Cas13Y). Each subtype can have different variants and according to the available information, RfxCas13d was the most effective and promising effector protein variant. Although there are some limitations to the use of these systems, further analysis and more information are needed. Prospects indicate that these systems hold promise as technologies for creating virus-resistant plants to be used in the field.

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