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RNA-Based Technologies for Functional Genomics in Plants

Concepts and Strategies in Plant Sciences

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ISSN 2662-3188 ISSN 2662-3196 (electronic)
Concepts and Strategies in Plant Sciences
ISBN 978-3-030-64993-7 ISBN 978-3-030-64994-4 (eBook)
https://doi.org/10.1007/978-3-030-64994-4

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Preface

Genome sequencing has revealed gene sequences of many plant species. Such genes, which also include non-coding ones, require functional interrogation for their underlying biological relevance and applications in crop trait improvement. Reverse genetics for functional genomics was fostered by high-throughput sequencing followed by the large-scale annotation of genes. After gene identification, functional characterization is mainly achieved by the creation of various gain- or loss-of-function mutants in plants. While the gain of gene function in plants is achieved through the expression of target genes driven by strong promoters such as cauliflower mosaic virus (CaMV) 35S, loss of gene function is commonly induced by ethane methyl sulfonate (EMS) mutagenesis or by T-DNA/transposon insertions which lead to mutated/truncated proteins with attenuated or null functions. All these approaches create mutations in non-specific manner and the desired target mutant will need to be fished from the pool of large mutant population.

More recent technologies are aimed at specific gene targeting to induce loss-of-function. Loss-of-function can be achieved by targeting either the DNA or RNA of a specific gene for alteration or silencing. To target a specific DNA (gene) in the genome, technologies such as zinc-finger nucleases (ZFNs), transcription activator-like effector nucleases (TALENs), meganucleases, and the clustered regularly interspaced short palindromic repeats (CRISPR)/CRISPR-associated nuclease (Cas) (CRISPR/Cas) system have been developed. ZFNs, TALENs, and meganucleases require complicated protein engineering before altering genes of interest. By contrast, CRISPR/Cas is an RNA-based DNA cleavage technology, making its application as simple as RNAi but more directional, effective, and diverse than traditional methods for creating genetic mutants.

Targeting the RNA of a given gene for silencing involves the use of RNA interference (RNAi), a great discovery in silencing genes post-transcriptionally. Over the years RNAi technology has undergone many developments, extending from a hairpin structure with inverted repeats to artificial miRNAs (amiRNAs). RNAi/small RNA-based gene silencing is widely used as a popular means to study gene function because it can target specific genes of known sequences to decipher their functions for the first time in a non-random manner. It can lead to gene silencing at either the transcriptional (target DNA methylation) or post-transcriptional levels (target RNA

cleavage or translational repression). In the genomics era, when the genomes of many plant and animal species have been sequenced, RNAi/small RNA-based gene silencing is extremely useful and has become a powerful approach to functional genomics, especially when genetic mutants are unavailable or not feasible.

Small RNAs, including miRNAs and small interfering RNAs (siRNAs), have emerged as key players in gene regulation during growth and development, in epigenetics, and in responses to various abiotic and biotic stresses by negatively regulating gene expression at the post-transcriptional level. Hundreds to thousands of miRNAs have been identified from different plant species. Functional genomics of these small RNA genes in the genome has become a new subject for technology development in plants and animals. Because of their small size, traditional technologies are not easily applicable to the study of small RNA function. Several specific technologies for functional genomics of small RNAs have been developed, such as miRNA target mimicry (TM) and short tandem target mimic (STTM) These technologies are powerful in inactivating small RNAs at the post-transcriptional level. Similarly, amiRNAs have been successfully used to downregulate target mRNAs and even miRNAs. Artificial/synthetic trans-acting siRNAs (atasiRNAs/syn-tasiRNAs) can also be used as an alternative to induce specific gene silencing in plants. It is feasible that CRISPR/Cas tool can be used to knock out multiple miRNAs or miRNA families by guide RNA (gRNA) multiplexing, as has been carried out for targeting multiple coding genes.

This book discusses key RNA-based technologies for functional genomics of plant coding and non-coding genes, using target mimics, RNAi, amiRNAs, and CRISPR/Cas approaches. This book focuses on how these RNA-based technologies have been developed, applied, and validated as essential technologies in plant functional genomics. These techniques will enable the users to functionally characterize genes and small RNAs through silencing, overexpression, and/or editing.

Houghton, USA — Guiliang Tang
Greater Noida, India — Sachin Teotia
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Dr. Xiaoqing Tang is an Associate Professor in the department of Biological Sciences, Michigan Technological University, USA. She did her Ph.D. from Weizmann Institute of Science, Israel, 2003. She has been involved in the characterization

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Chapter 1
Artificial Small RNAs for Functional Genomics in Plants

Adriana E. Cisneros, Ainhoa de la Torre-Montaña, Tamara Martín-García, and Alberto Carbonell

Abstract RNA interference (RNAi) is based on the sequence-specific degradation of target RNAs by highly complementary small RNAs (sRNAs), which can be engineered to selectively target genes of interest. In plants, artificial microRNAs (amiRNAs) and artificial/synthetic trans-acting small interfering RNAs (atasi/syntasiRNAs) are the two main classes of artificial small RNAs (art-sRNAs). Art-sRNAs are refined, highly specific, selective, and potent RNAi tool that has been extensively used in gene function studies and for crop improvement. Here we describe the biogenesis and function of art-sRNAs, and how they are designed and used to study the function of plant genes.

Keywords Artificial small RNA · Functional genomics · Plants · RNA silencing · Artificial microRNA · Artificial tasiRNA · Synthetic tasiRNA

1.1 Introduction

In the current genomic era, the use of high-throughput sequencing technologies has allowed the identification of the genes of a large number of organisms, including model and crop plants (Parinov and Sundaresan 2000; Morozova and Marra 2008). In this context, one of the main challenges of modern plant biology is the characterization of the function of the genes of relevant plant species. Typically, once a gene has been identified, its functional characterization is assessed by the generation of either gain- or loss-of-function mutant plants with enhanced or reduced/null gene activity, respectively (Kuromori et al. 2009). Historically, gain-of-function mutants have been generated mainly through the transgenic overexpression of the target gene using potent constitutive promoters such as *Cauliflower mosaic virus* (CaMV) 35S (Weigel et al. 2000), while loss-of-function mutants have been obtained through ethane methyl sulfonate (EMS) mutagenesis (Kim et al. 2006) or by T-DNA insertion

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© Springer Nature Switzerland AG 2021

G. Tang et al. (eds.), *RNA-Based Technologies for Functional Genomics in Plants*,
Concepts and Strategies in Plant Sciences, https://doi.org/10.1007/978-3-030-64994-4_1

(Azpiroz-Leehan and Feldmann 1997). All these approaches have been extensively used for decades, despite their randomness in the gene targeting process.

In recent years, efforts have seeked to develop technologies for more controlled and efficient gene targeting, mainly to generate loss-of-function mutant plants. Indeed, a plethora of tools for targeting either the DNA or the RNA of a given gene have been developed and applied successfully to plants in gene function studies. DNA targeting tools include technologies such as zinc-finger nucleases (ZFNs), transcription activator-like effector nucleases (TALENs), meganucleases, and the clustered regularly interspaced short palindromic repeats [CRISPR/CRISPR-associated nuclease 9 (Cas9) system] (Teotia et al. 2016). RNA targeting tools have exploited endogenous sRNA-directed silencing pathways controlling gene expression, stress responses, and genome integrity. Classic RNA interference (RNAi) technologies such as virus-induced gene silencing (VIGS) or hairpin-based silencing rely on the expression of double-stranded RNA (dsRNA) or dsRNA-like precursors including sequences corresponding to the target transcript to trigger small interfering RNA (siRNA) production to silence complementary target sequences (Ossowski et al. 2008; Baykal and Zhang 2010). Despite their massive use, these strategies are not considered highly specific as the large populations of siRNAs generated from dsRNA precursors might accidentally target cellular transcripts with high sequence complementarity to that of certain siRNAs. More recently, a series of more refined "second-generation RNAi" strategies with high specificity have been developed and applied successfully in gene function studies and crop improvement (Carbonell 2017a). These strategies are based on the expression of plant artificial sRNAs (art-sRNAs). Here, we describe what art-sRNAs are, and how they are designed, produced, and used in gene function studies in plants.

1.2 Artificial sRNAs (Art-sRNAs)

Art-sRNAs are 21-nucleotide sRNAs designed to selectively target one or several RNAs with high specificity and efficacy, by exploiting endogenous sRNA pathways. The two main classes of plant art-sRNAs are described next.

1.2.1 Artificial microRNAs (amiRNAs)

In plants, microRNAs (miRNAs) arise from miRNA transcripts with imperfect self-complementary foldback structures transcribed from endogenous *MIRNA* genes (Fig. 1.1a). These miRNA foldbacks are processed by DICER-LIKE1 (DCL1) to generate miRNA duplexes. One of the strands of the duplex, the miRNA guide strand, is selectively loaded into a protein of the ARGONAUTE (AGO) family based on the identity of the 5' nucleotide of the sRNA and/or other sequence and structural

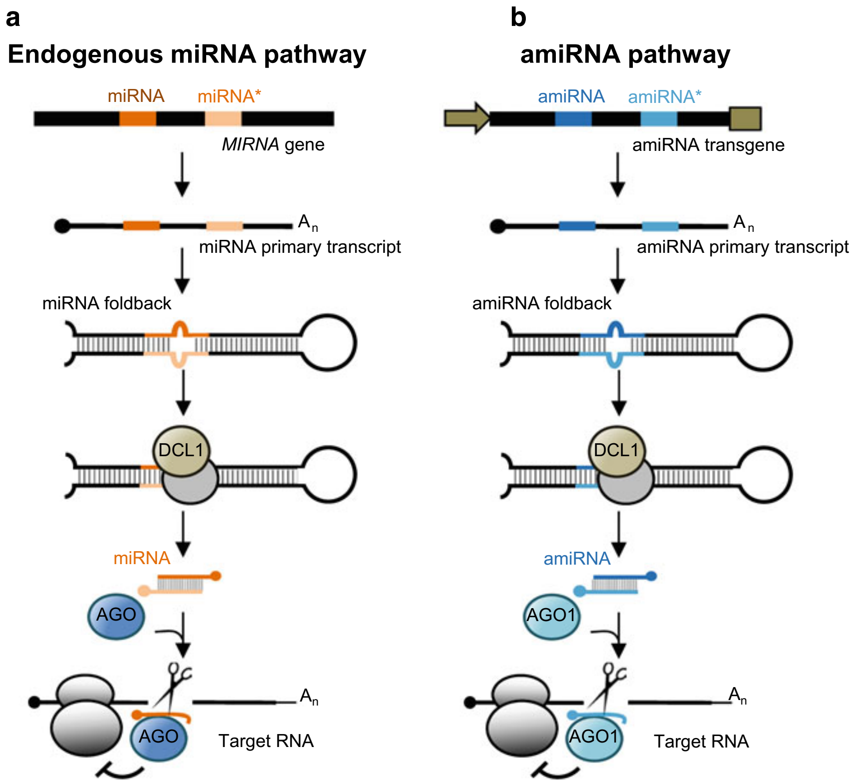

Fig. 1.1 Endogenous and artificial miRNA pathways in plants. Left, endogenous miRNA pathway. A *MIRNA* gene is represented in black with native miRNA/miRNA* sequences in dark and light orange, respectively. Right, the amiRNA pathway. An amiRNA transgene is introduced into plants, and includes exogenous promoter and terminator sequences (gold arrow and box, respectively), and the sequence of a plant miRNA precursor (in black) in which the original miRNA/miRNA* sequences have been substituted by the amiRNA/amiRNA* sequences (in dark and light blue, respectively). The transgene expresses an amiRNA primary transcript which is processed into an amiRNA foldback. A rationale amiRNA design requires that the amiRNA foldback preserves the original secondary structure of the endogenous precursor, and that the amiRNA guide strand contains a 5'U nucleotide to favor its association with AGO1 to silence highly complementary transcripts

features of the sRNA duplex and the AGO (Takeda et al. 2008; Mi et al. 2008; Montgomery et al. 2008a; Zhu et al. 2011; Zhang et al. 2014b), while the other strand (the star *) is usually degraded. The miRNA guides the AGO to bind and silence highly sequence complementary RNAs either by direct slicing or by repressing their translation (Fig. 1.1a) (Bologna and Voinnet 2014; Carbonell 2017b).

Artificial miRNAs (amiRNAs) are typically expressed *in planta* from transgenes including a miRNA precursor in which the original miRNA/miRNA* sequences have been substituted by the amiRNA/amiRNA* sequences (Fig. 1.1b). The amiRNA transgene is transcribed into a primary transcript that follows the canonical miRNA

biogenesis pathway. Importantly, amiRNAs are designed to contain a 5' U that favors AGO1 loading and subsequent silencing of cognate transcripts (Fig. 1.1b) (Carbonell 2017a). Typically, amiRNAs have been used to target a single target transcript, although other methodologies for co-expressing multiple amiRNAs from a single construct have also been reported. These include the expression of multiple amiRNAs from different precursors in tandem (Kung et al. 2012; Liang et al. 2012; Zhang et al. 2018a) or polycistronic precursors (Fahim et al. 2012; Kis et al. 2016).

1.2.2 Artificial/Synthetic Trans-Acting Small Interfering RNAs (atasi/syn-tasiRNAs)

Trans-acting siRNAs (tasiRNAs) are a particular subclass of plant sRNAs that arise from transcripts of *TAS* genes in *Arabidopsis thaliana.* The biogenesis pathway of endogenous tasiRNA is initiated by the cleavage of a *TAS* transcript by a miRNA/AGO complex, which triggers the recruitment of RNA-DEPENDENT RNA POLYMERASE6 (RDR6) to synthesize dsRNA from one of the cleavage products (Fig. 1.2a) (Allen et al. 2005; Rajagopalan et al. 2006). The dsRNA is sequentially processed by DCL4 into 21 nucleotide (nt) tasiRNA duplexes in register with the miRNA-guided cleavage site (Yoshikawa et al. 2005; Montgomery et al. 2008b). As for miRNAs, the guide strand is selectively loaded into an AGO protein to direct the silencing of highly sequence complementary RNAs (Fig. 1.2a) (Yoshikawa et al. 2005; Deng et al. 2018).

Artificial/synthetic tasiRNAs (atasiRNAs/syn-tasiRNAs) are produced in plants expressing a transgene containing a *TAS* precursor in which a subset of the native tasiRNA sequences has been substituted by several syn-tasiRNA sequences in tandem (Fig. 1.2b) (Zhang 2014; Carbonell 2017a). The atasiRNA/syn-tasiRNA transgene is transcribed into a primary transcript that follows the canonical tasiRNA biogenesis pathway. AtasiRNAs/syn-tasiRNAs, as described for amiRNAs, are designed to contain a 5' U to favor association with AGO1 and lead to the silencing of one or multiple highly sequence complementary transcripts (Fig. 1.2b) (Carbonell 2017a). Typically, syn-tasiRNA constructs are used to co-express multiple syn-tasiRNAs targeting different sites in the same transcript (de la Luz Gutierrez-Nava et al. 2008) or transcripts from different genes (Carbonell et al. 2014, 2019a, b; Chen et al. 2016; Carbonell and Daros 2017).

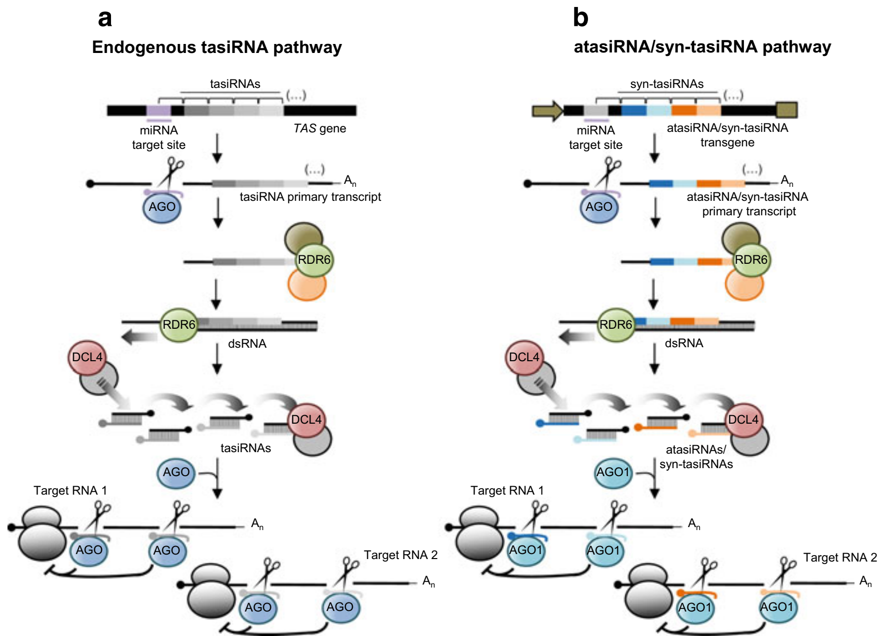

Fig. 1.2 Endogenous and artificial/synthetic tasiRNA pathways in plants. **a** The tasiRNA pathway. **b** The artificial/synthetic tasiRNA pathway. An atasiRNA/syn-tasiRNA transgene, containing a plant *TAS* precursor in which a subset of the original tasiRNA sequences has been substituted by several syn-tasiRNA sequences in tandem, is introduced into plants to express a syn-tasiRNA primary transcript. An endogenous miRNA cleaves this primary transcript, a process that triggers the recruitment of RDR6 complexes to synthesize a dsRNA from one of the cleavage products. DCL4 processes the dsRNA into phased tasiRNA duplexes in 21 nt register with the miRNA cleavage site. Syn-tasiRNA guide strands with a 5'U are incorporated into AGO1 to direct specific silencing of sequence unrelated target transcripts at one or multiple sites

1.3 Design, Production, and Validation of Art-sRNA Constructs

Despite the extensive use of art-sRNAs during the last decade, the design, production, and validation of art-sRNA constructs for plants has been a tedious process until very recently. The development of a series of high-throughput methodologies to generate art-sRNA constructs in a time- and cost-effective manner allows the efficient use of these tools in gene functional studies.

1.3.1 Design of Plant Art-sRNAs

Plant art-sRNAs are designed to be highly effective and highly specific with the help of automated web tools such as WMD3 (from Web MicroRNA Designer 3, http://wmd3.weigelworld.org/cgi-bin/webapp.cgi) (Ossowski et al. 2008), amiRNA Designer (http://www.cs.put.poznan.pl/arybarczyk/AmiRNA) (Mickiewicz et al. 2016), micro RNA Designer (http://www.smallrna.mtu.edu/Tang_Website/submit.htm), and P-SAMS (from Plant Small RNA Maker Suite, http://p-sams.carringtonlab.org/) (Fahlgren et al. 2016). To account for high efficacy, these tools generally design art-sRNAs with extensive sequence complementarity with the target RNA. Despite the rules governing productive sRNA/target RNA interactions are not fully understood, it is well known that (i) the degree of silencing induced by an art-sRNA positively correlates with the degree of base-pairing between the sRNA and the target RNA (Liu et al. 2014), and (ii) mismatches included in the sRNA "seed region" (nucleotides 2–14) drastically decrease the activity of the sRNA (Schwab et al. 2006; Fahlgren and Carrington 2010). In any case, the efficacy of a given art-sRNA is difficult to predict, as the in vivo accessibility of target sites can be limited if they form highly structured conformations or if they are occupied by RNA-binding proteins. To account for high specificity, design tools assess the specificity of each art-sRNA in a given plant species by analyzing all possible base-pairing interactions between the candidate art-sRNA and the complete set of cellular transcripts of this species. Thus, the transcriptome of this particular species must be available, and, ideally, well-annotated.

To date, P-SAMS is the only web tool allowing for the design of the two classes of plant art-sRNAs: amiRNAs and atasiRNAs/syn-tasiRNAs, through its P-SAMS amiRNA Designer and P-SAMS Syn-tasiRNA Designer applications, respectively (Fahlgren et al. 2016). Briefly, P-SAMS has a user-friendly interface combined with a wizard-assisted navigation through simple questions that the user answers during the design process. An FAQ page addresses usual questions and contains video tutorials describing the different types of designs. Job times for designs are relatively short compared to other tools. For example, typical median job time for single-targeting amiRNA design is approximately 3 min. Results are displayed on-screen and include the sequence of up to three "optimal" art-sRNA and/or up to three "suboptimal" art-sRNAs if off-targets are predicted or not, respectively, as well as the sequence of the two oligonucleotides required for cloning into compatible "b/c" vectors (see section below). If the off-target filtering is activated, P-SAMS starts by cataloguing all target sites not containing a 15 nucleotide sequence from positions 6–20 perfectly matching a transcript not included in the input set. Then, an art-sRNA with the following sequence features is designed to target each target site from the input transcript: (i) the art-sRNA contains a 5'U nucleotide that favors AGO1 association, (ii) position 19 of the art-sRNA is a C to generate a star strand with an AGO-non preferred 5'G, and (iii) position 21 of the art-sRNA does not base-pair with the target transcript to reduce chances of triggering transitivity.

1.3.2 Generation of Art-sRNA Constructs

To generate an art-sRNA construct, a DNA fragment corresponding to the amiRNA insert has to be introduced in a plasmid including the plant precursor sequence flanked by regulatory promoter and terminator sequences. The selection of an appropriate art-sRNA precursor to clone and express the art-sRNA is a critical step, as it will actually influence both the cloning procedure and the in vivo activity of the art-sRNA. Moreover, it is recommended to use an evolutionary conserved precursor that most likely will be accurately processed in a large number of plant species (Carbonell 2017a).

Regarding the cloning, the generation of art-sRNA constructs can be a tedious process of several days. For example, classic methodologies for amiRNA cloning involved a large number of steps such as various PCRs, gel purifications, restriction and ligation reactions, subcloning, etc. (Schwab et al. 2006; Warthmann et al. 2008; Molnar et al. 2009). One of the reasons is that some of the amiRNA precursors used were excessively long, and thus not well adapted for an easy cloning. More recent technologies have been developed for high-throughput cloning of art-sRNAs mainly by reducing the number of steps during cloning (Chen et al. 2009; Yan et al. 2011, 2012; Carbonell et al. 2014; Hu et al. 2014; Li et al. 2014b; Luo et al. 2018; Carbonell 2019a). For example, the *Ath-MIR390a* and *Osa-MIR390* precursors from the well-conserved *MIR390* family were selected to clone and express amiRNAs in eudicot and monocot species, respectively, due to their short size compared to other miRNA precursors, that facilitated the synthesis and cloning of the amiRNA insert in zero-background cloning/expression "B/c" vectors containing a modified version of the *MIR390* precursor interrupted by a *ccd*B cassette flanked by two inverted *Bsa*I sites (Carbonell et al. 2014, 2015). AmiRNA inserts are obtained by annealing two partially complementary and overlapping oligonucleotides containing the amiRNA/stem-loop/amiRNA* region, and present 4 nucleotide 5' overhangs compatible with those resulting from the *Bsa*I digestion of the "B/c" vector. AmiRNA inserts are directly cloned into "B/c" vectors in a 5 min digestion–ligation reaction in the presence of *Bsa*I and T4 DNA ligase (for a detailed description see [Carbonell 2019a]). A similar strategy was developed for generating atasi/syn-tasiRNA constructs (Carbonell et al. 2014; Carbonell 2019a). The development of these types of high-throughput methodologies to generate art-sRNA constructs should definitely facilitate the use of the art-sRNA technology in functional genomics studies.

1.3.3 In Vivo Validation of Art-sRNA Constructs

Despite a thorough web tool-assisted design and subsequent cloning into a well-established expression vector, the correct activity of a given art-sRNA construct cannot be taken for granted. A first step to validate in vivo an art-sRNA construct

is to check that the art-sRNA accumulates *in planta* as a single sRNA species of the correct size. This can be evaluated by combining Northern blot hybridization with deep sequencing analysis (Carbonell et al. 2014, 2015). The accuracy of the processing of the art-sRNA precursor typically results in the accumulation of the art-sRNA as a single species in Northern blot analysis, and in the overrepresentation in sRNA libraries of reads corresponding to the art-sRNA compared to reads mapping to other precursor positions. In the case of syn-tasiRNA constructs including multiplexed syn-tasiRNAs, sRNA libraries are used to confirm the correct phasing of syn-tasiRNAs (Carbonell et al. 2014). Indeed, a rapid assessment of in vivo art-sRNA accumulation can be done by transiently expressing the art-sRNA construct in *Nicotiana benthamiana* leaves (Yu and Pilot 2014).

The second validation step is to assess the art-sRNA efficacy in silencing its corresponding target(s). Ideally, the efficacy of the art-sRNA can be inferred visually if target silencing leads to an obvious phenotype, which may be quantitative. If not, target gene silencing can be analyzed by measuring target RNA levels by quantitative RT-PCR, and art-sRNA cleavage sites are mapped by 5' RLM-RACE (Schwab et al. 2006). Alternatively, genome-wide transcriptome profiling through RNA sequencing can be used both to quantify target RNA accumulation and art-sRNA specificity (Carbonell et al. 2015). Very recently, degradome analysis has also served to check sRNA specificity (Singh et al. 2019), although through an MiRNA-Induced Gene Silencing (MIGS) strategy (Felippes et al. 2012), where the specificity of generated siRNAs is not controlled (Carbonell 2019b). In any case, it is important to consider that art-sRNA constructs can be easily screened and validated in *N. benthamiana* transient assays to select the most effective for stable expression in transgenic plants (Yu and Pilot 2014; Carbonell et al. 2019a, b). Alternatively, amiRNA efficacy can be assessed in epitope-tagged protein-based amiRNA (ETPamiR) screens, where target transcript encoding epitope-tagged proteins are co-expressed with amiRNA candidates in protoplasts (Li et al. 2013, 2014a).

1.4 Examples of Art-SRNAs Used in Gene Function Studies in Plants

Art-sRNAs, mainly amiRNAs, have been extensively used to silence genes in a wide range of plant species, from model plants to ornamentals and crops. A list of the precursors successfully used to express art-sRNAs in different plant species is presented in Table 1.1.

Despite art-sRNAs have been widely used for crop improvement, including the generation of antiviral resistance, a major use of this technology has focused on silencing plant genes in order to study their function. Here, we will describe just a few representative examples on how art-sRNAs can accelerate gene function discovery.

Table 1.1 Examples of uses of artificial sRNA precursors in plants

Artificial sRNA	Plant Species	Common name	Precursor used	References
amiRNA	*Arabidopsis thaliana*	Thale cress	*Ath-MIR159a* *Ath-MIR159b* *Ath-MIR164a* *Ath-MIR169d* *Ath-MIR171a* *Ath-MIR172a* *Ath-MIR319a* *Ath-MIR390a* *Ath-MIR395a*	Niu et al. (2006) Eamens et al. (2011) Alvarez et al. (2006) Liu et al. (2010) Qu et al. (2007) Schwab et al. (2006) Schwab et al. (2006) Montgomery et al. (2008a) Liang et al. (2012)
	Brachypodium distachyon	Purple false brome	*Osa-MIR390-AtL* *Osa-MIR528*	Carbonell et al. (2015) Smertenko et al. (2020)
	Catharanthus roseus	Madagascar periwinkle	*Ath-MIR319a*	Li et al. (2013)
	Chlamydomonas reinhardtii	Green algae	*Cre-MIR1157* *Cre-MIR1162*	Molnar et al. (2009) Zhao et al. (2009)
	Corchorus olitorius	Jute mallow	*Ath-MIR319a*	Shafrin et al. (2015)
	Fragaria Vesca	Strawberry	*Fve-MIR166*	Li et al. (2019)
	Glycine max	Soybean	*Ath-MIR319a*	Melito et al. (2010)
	Helianthus annuus	Sunflower	*Ath-MIR319a*	Li et al. (2013)
	Hordeum vulgare	Barley	*Hvu-MIR171*	Kis et al. (2016)
	Lemna minor	Duckweed	*Lgi-MIR166a*	Canto-Pastor et al. (2015)
	Malus domestica	Apple	*Mdo-MIR156h*	Charrier et al. (2019)
	Medicago sativa	Alfalfa	*Ath-MIR319a*	Verdonk and Sullivan (2013)

(continued)

Table 1.1 (continued)

Artificial sRNA	Plant Species	Common name	Precursor used	References
	Nicotiana benthamiana	–	*Ath-MIR159a* *Ath-MIR319a* *Ath-MIR390a* *Ghb-MIR169a* *Hvu-MIR171* *Vvi-MIR166f* *Vvi-MIR319e*	Mitter et al. (2016) Li et al. (2013) Montgomery et al. (2008a) Ali et al. (2013) Kis et al. (2016) Roumi et al. (2012) Castro et al. (2016)
	Nicotiana tabacum	Tobacco	*Ath-MIR159a* *Ath-MIR164b* *Ath-MIR319a* *Sly-MIR159* *Sly-MIR168a*	Mitter et al. (2016) Alvarez et al. (2006) Vu et al. (2013) Vu et al. (2013) Vu et al. (2013)
	Marchantia polymorpha	Liverwort	*Mpo-MIR160*	Flores-Sandoval et al. (2016)
	Medicago truncatula	Barrelclover	*Mtr-MIR159b*	Devers et al. (2013)
	Oryza sativa	Rice	*Osa-MIR528*	Warthmann et al. (2008)
	Petunia hybrida	Garden petunia	*Ath-MIR319a*	Guo et al. (2014)
	Phaeodactylum tricornutum	Marine diatom	*Ath-MIR319a*	Kaur and Spillane (2015)
	Physcomitrella patens	Spreading earthmoss	*Ath-MIR319a*	Khraiwesh et al. (2008)
	Populus trichocarpa	Poplar	*Ptc-MIR408*	Shi et al. (2010)
	Solanum lycopersicum	Tomato	*Ath-MIR159a* *Ath-MIR164a* *Ath-MIR319a* *Ath-MIR390a* *Sly-MIR159* *Sly-MIR168a*	Zhang et al. (2011) Alvarez et al. (2006) Fernandez et al. (2009) Carbonell et al. (2019a) Vu et al. (2013) Vu et al. (2013)
	Solanum melongena	Eggplant	*Ath-MIR319a*	Toppino et al. (2011)

(continued)

Table 1.1 (continued)

Artificial sRNA	Plant Species	Common name	Precursor used	References
	Solanum tuberosum	Potato	*Ath-MIR168a* *Ath-MIR319a*	Bhagwat et al. (2013) Wyrzykowska et al. (2016)
	Vitis vinifera	Grape	*Ath-MIR319a*	Jelly et al. (2012)
	Triticum aestivum	Wheat	*Osa-MIR395*	Fahim et al. (2012)
	Whitania somnifera	Ashwagandha	*Ath-MIR159a*	Singh et al. (2016)
	Zea mays	Maize	*Ath-MIR319a*	Li et al. (2013)
atasiRNA/ syn-tasiRNA	*Arabidopsis thaliana*	Thale cress	*Ath-TAS1a* *Ath-TAS1c*	Felippes and Weigel (2009) de la Luz Gutierrez-Nava et al. (2008)
	Nicotiana benthamiana	–	*Ath-TAS1c* *Ath-TAS3a*	Montgomery et al. (2008b) Montgomery et al. (2008a)
	Solanum lycopersicon	Tomato	*Ath-TAS1c*	Carbonell et al. (2019a)

1.4.1 Artificial MiRNAs

Besides their extensive biotechnological use in crop improvement (Kamthan et al. 2015), amiRNAs have been broadly used to silence plant genes in functional studies in both model and crop plants (Sablok et al. 2011; Tiwari et al. 2014) (see Table 1.2).

1.4.1.1 Silencing of Coding Genes

A major problem to assign gene functions in plants is the presence of large gene families, which cause functional genetic redundancies and partial or complete functional overlap among closely related genes, as observed in the Arabidopsis genome (2000). Indeed, this may be the reason for the absence of visible phenotypes in single mutants. In this scenario, and because amiRNAs can target both single and multiple gene family members, amiRNA-based tools for screening the functionally redundant gene space were developed. First, a computationally derived library of 22,000 genome-wide family-specific amiRNAs was synthesized in multiple sub-libraries, each targeting defined functional protein classes (Hauser et al. 2013). For example, this amiRNA collection was used to encover novel morphological seed germination mutants for amiRNAs targeting zinc-finger homeodomain transcription factors

Table 1.2 Examples of uses of amiRNAs to study gene function in plants

Plant species	Target(s)[a]	Gene function studied	References
Arabidopsis thaliana	576 transcription factor genes	Redundancy in transcription factors	Jover-Gil et al. (2014)
	All *A. thaliana* protein-coding genes	Functional redundancy of Arabidopsis genes	Hauser et al. (2013)
		Identification of genes involved in CO_2 and abscisic acid responses	Hauser et al. (2019)
	Homologous genes with subclades of transporter families	Transport of signaling molecules	Zhang et al. (2018b)
	Ath-ADK	Adenosine kinase role in cytokinin interconversion	Schoor et al. (2011)
	Ath-AGP6/11	Role of arabinogalactan proteins in pollen development	Coimbra et al. (2009)
	Ath-CaM1	Senescence and abscisic acid response	Dai et al. (2018)
	Ath-CH42	Movement of the silencing signal	de Felippes et al. (2011)
	Ath-CHS	Asymmetric 22-nt miRNA role trigger widespread RNA silencing	McHale et al. (2013)
	AthCIPK16	Identification of a protein kinase involved in Na + exclusion	Roy et al. (2013)
	Ath-CKB, *Ath-ELF3*, *Ath-GI*, *Ath-ZTL*	Circadian clock regulation	Kim and Somers (2010)
	Ath-ERF102, *Ath-ERF104*	Cold stress	Illgen et al. (2020)
	Ath-cpHSC70-1/2	Involvement of heat shock proteins in chloroplast development	Latijnhouwers et al. (2010)

(continued)

Table 1.2 (continued)

Plant species	Target(s)[a]	Gene function studied	References
	Ath-FAD2, *Ath-FAE1*, *Ath-FATB*	Seed oil composition content	Belide et al. (2012)
	Ath-FT	Molecular mechanisms of flowering	Schwartz et al. (2009) Yeoh et al. (2011)
	Ath-H2AZ	Role of the SWR1 complex in flowering and development	Choi et al. (2007)
	Ath-IPMI-SSU1	Role of *Ath-IPMI-SSU1* in growth and development	Imhof et al. (2014)
	Ath-LNP1, Ath-LNP1/2	ER cisternae formation	Kriechbaumer et al. (2018)
	Ath-MAS2	Involvement of *MAS2* in 45S ribosomal DNA silencing	Sánchez-García et al. (2015)
	Ath-MIR159, Ath-MIR164	Specific functions of different amiRNA family members	Eamens et al. (2011)
	Ath-MIR408	Functional characterization of *MIR408*	Zhang and Li (2013)
	Ath-MYB14	Identification of genes involved in freeze tolerance	Chen et al. (2013)
	Ath-NB-LRR	Role of NB-LRR in autoinmune responses like hybrid necrosis	Bomblies et al. (2007)
	Ath-PHB, *Ath-REV*	microRNA sorting into AGOS	Zhang et al. (2014b)
	Ath-PP2AA1/2/3	Identification of phosphatase components in polar targeting of PIN auxin transport proteins	Michniewicz et al. (2007)
	Ath-PPPC4	New function in salt tolerance	Wang et al. (2012)

(continued)

Table 1.2 (continued)

Plant species	Target(s)[a]	Gene function studied	References
	Ath-PPR4	Trans-splicing of *rps12* chloroplast transcripts	Lee et al. (2019)
	Ath-SAUR19-24	Cell expansion	Spartz et al. (2012)
	Ath-SEP3	Role of DNA polymerase δ in the deposition of epigenetic marks, development, and flowering	Iglesias et al. (2015)
	Ath-snRK2	Involvement of SnRK2s in BIN2-modulated abscisic acid responses	Cai et al. (2014)
	Ath-TAS1c	tasiRNA biogenesis	Cuperus et al. (2010)
	Ath-TAS1c-A388U	AGO2-mediated target slicing	Carbonell et al. (2012)
	Ath-TAS2	tasiRNA biogenesis	Yoshikawa et al. (2013)
	Ath-U11/U12-31 K	Role *of Ath-U11/U12-31 K* in U12 intron splicing and plant development	Kim et al. (2010)
	Ath-U11/u12-65 K	Role of *Ath-U11/u12-65 K* in U12 intron splicing and plant development	Jung and Kang (2014)
	Ath-XCT	*RESISTANCE TO POWDERY MILDEW8.1*-based immunity	Xu et al. (2017)
	GFP	Pollen development	Grant-Downton et al. (2013)
		sRNA movement	Slotkin et al. (2009)
Brachypodium distachyon	*Bdi-MAP20*	Metaxylem pit development and drought recovery	Smertenko et al. (2020)

(continued)

Table 1.2 (continued)

Plant species	Target(s)[a]	Gene function studied	References
	Bdi-GT43B2	Xylan biosynthesis and seedling survival	Petrik et al. (2020)
Chlamydomonas reinhardtii	*Chr-CDPK3*	Regulation of flagellar biogenesis by a calcium-dependent protein kinase	Liang and Pan (2013)
	Chr-HSF1	Identification of genes involved in thermotolerance	Schmollinger et al. (2010)
	Chr-HydA1, *Chr-HydA2*, *Chr-HYD3*	Hydrogenase activity	Godman et al. (2010)
	Chr-MDAR1	Tolerance to photooxidative stress	Yeh et al. (2019)
	Chr-PEPC1/2	Role in fatty acid accumulation	Wang et al. (2017)
Chysanthemum morifolium	*Cmo-BBX8*	Flowering time	Wang et al. (2020b)
Glycine max	*Gma-Rhg1*	Identification of genes involved in resistance to cyst nematode	Melito et al. (2010)
	Gma-tRF001, *Gma-tRF003*	Nodulation regulation by rhizobial tRFs	Ren et al. (2019)
Medicago truncatula	*Mtr-FLOT2*, *Mtr-FLOT3*, *Mtr-FLOT4*	Flotillin requirement for bacterial infection	Haney and Long (2010)
Nicotiana benthamiana	–	microRNA sorting into AGOs	Zhang et al. (2014b)
	Ath-DRB1	microRNA sorting	Eamens et al. (2009)
	Nbe-SACPD-A/B, *Nbe-SACPD-A/B/C*, *Nbe-SACPD-C*	Ovule development	Zhang et al. (2014a)
	Nbe-siPPase	Involvement of viroid-derived sRNAs in symptom development	Eamens et al. (2014)
	Ppy-LUC	Functionality of intron-derived miRNAs	Shapulatov et al. (2018)
		Secondary siRNA production	Manavella et al. (2012)

(continued)

Table 1.2 (continued)

Plant species	Target(s)[a]	Gene function studied	References
Nicotiana tabacum	*Nta-CHS*	Hairy root metabolism	Hidalgo et al. (2017)
	Nta-FLS	Resistance to insects	Misra et al. (2010)
	Nta-siPPase	Involvement of viroid-derived sRNAs in symptom development	Eamens et al. (2014)
Oriza sativa	*Osa-A2*	Grain yield, shoot growth, and nitrogen level	Loss Sperandio et al. (2020)
	Osa-Eui1	Elongation of the uppermost internode at heading stage	Warthmann et al. (2008)
	Osa-GLP2-1	Seed dormancy	Wang et al. (2020a)
	Osa-HDAC1, *Osa-HDAC2* *Osa-HDAC3*	Histone deacetylation	Hu et al. (2009)
	Osa-PLL3, *Osa-PLL4*	Pollen development in rice panicles	Zheng et al. (2018)
	Osa-Spl11	Lesion formation in the absence of pathogen	Warthmann et al. (2008)
Physcomitrella patens	*Ppa-FtsZ2-1, Ppa-GNT1*	Chloroplast division, miRNA processing	Khraiwesh et al. (2008)
Populus tomentosa	*Ptr-SS3*	Secondary growth	Li et al. (2020)
Populus trichocarpa	*Ptr-PAL2/4/5, Ptr-PAL1/3*	Identification of phenylalanine ammonia lyase (PAL) genes	Shi et al. (2010)
Solanum lycopersicum	*Sly-CLC-b, Sly-RPS3a*	Cleavage of endogenous transcripts by viroid-derived sRNAs	Adkar-Purushothama et al. (2017)
Solanum melongene	*Sme-TAF10* *Sme-TAF13*	Male sterility	Toppino et al. (2011)
Solanum tuberosum	*Stu-CBP89*	Molecular mechanisms of drought tolerance	Pieczynski et al. (2013)
	Stu-PPO1, Stu-PPO2, Stu-PPO3, Stu-PPO2/3, Stu-PPO2/3/4, Stu-PPO1/2/3/4	Individual contribution of different PPO genes in total PPO protein activity	Chi et al. (2014)
Whitania somnifera	*Wso-SGTL1/2/3*	Role of sterol glycosyltransferases, antibacterial resistance	Singh et al. (2016)

(continued)

Table 1.2 (continued)

Plant species	Target(s)[a]	Gene function studied	References
	Wso-CYP85A69	Role in triterpenoids biosynthesis	Sharma et al. (2019)
Zea mays	*Zma-ZCN*	Molecular mechanisms of flowering	Meng et al. (2011)

[a]*ADK, ADENOSINE KINASE*; *AGP, ARABINOGALACTAN PROTEIN*; *BBX8, B-BOX PROTEIN8*; *CaM1, CALMODULIN1*; *CBP89, CAP-BINDING PROTEIN89*; *CDPK3, CALCIUM-DEPENDENT PROTEIN KINASE3*; *CH42, CHLORINA42*; *CHS, CHALCONE SYNTHASE*; *CIPK16, CBL-INTERACTING PROTEIN KINASE16*; *CKB, CASEIN KINASE II BETA*; *CLC-b, CHLORIDE CHANNEL PROTEIN b*; *cpHSC70, CHLOROPLASTIC HEAT SHOCK PROTEIN70*; *DRB1, DOUBLE-STRANDED RNA-BINDING PROTEIN1*; *ELF3, ELONGATION FACTOR3*; *ERF102/104, ETHYLENE RESPONSE FACTOR102/104*; *Eui1, ELONGATED UPPERMOST INTERNODE1*; *FAD2, FATTY ACID DESATURASE2*; *FAE1, FATTY ACID ELONGASE1*; *FATB, FATTY ACYL-ACP THIOESTERASE B*; *FLOT, FLOTILLIN-LIKE*; *FLS, FLAVONOL SYNTHASE*; *FT, FLOWERING LOCUS T*; *FTsZ2,* gene encoding a plastidial division protein; *GFP, GREEN FLUORESCENT PROTEIN*; *GI, GIGANTEA*; *GLP2-1, GERMIN-LIKE PROTEIN2-1*; *GNT1*, gene encoding an N-acetylglucosaminyltransferase; *GT43B2*, ortholog of wheat GT43-4 xylan synthase scaffolding protein; *H2AZ,* nucleosomal histone H2A variant Z; *HDAC, HISTONE DEACETYLASE*; *HSF1, HEAT SHOCK FACTOR1*; *HYD3, HYDROGENASE-LIKE PROTEIN3*; *HydA1/A2, FeFe-HYDROGENASEA1/A2*; *IPMI-SSU1, ISOPROPYLMALATE ISOMERASE SMALL SUBUNIT1*; *LNP1/2, LUNAPARK1/2*; *LUC, LUCIFERASE*; *MAP20, MICROTUBULE-BINDING PROTEIN20*; *MAS2, MORPHOLOGY OF AGO1-52 SUPPRESSED2*; *MDAR1, MONODEHYDROASCORBATE REDUCTASE1*; *MYB14, MYELOBLASTOSIS14*; *NB-LRR, NUCLEOTIDE-BINDING-SITE-LEUCINE-RICH-REPEAT*; *PAL, PHENYLALANINE AMMONIA-LYASE*; *PEPC1/2, PHOSPHOENOLPYRUVATE CARBOXYLASE1/2*; *PHB, PHAVOLUTA*; *PLL, PECTATE LYASE-LIKE; PP2A, PROTEIN PHOSPHATASE2A*; *PPO, POLYOHENOL OXIDASE*; *PPPC4, BACTERIAL-TYPE PHOSPHOENOLPYRUVATE CARBOXYLASE*; *PPR4, PENTATRICOPEPTIDE REPEAT*; *Rhg1,* gene conferring resistance to *Heterodera glycines*; *REV, REVOLUTA*; *RPS3a, RIBOSOMAL PROTEIN S3a*; *SACPD, STEAROL-ACYL CARRIER PROTEIN DESATURASE*; *SAUR, SMALL AUXIN UP RNA*; *SEP3, SEPALLATA3*; *SGTL, STEROL CLYCOSYLTRANSFERASE*; *siPPase, SOLUBLE INORGANIC PIROPHOSPHATASE*; *snRK2, snf1-RELATED KINASE2S*; *SPL11, SPOTTED LEAF11*; *SS3, SUCROSE SYNTHASE3*; *TAF, TBP-ASSOCIATED FACTORS*; *TAS, TRANS-ACTING SIRNA*; *tRF, RHIZOBIAL tRNA-DERIVED FRAGMENT*; *XCT, XAP5 CIRCADIAN TIMEKEEPER; ZCN, ZEA MAYS CENTRORADIALIS*; *ZTL, ZEITLUPE*

(Hauser et al. 2013), and more recently, to generate a seed resource for screening functional redundant genes and isolation of new mutants impaired in carbon dioxide and abscisic acid (Hauser et al. 2019). Another effort to simplify the analysis of gene function between gene families was the generation of a collection of amiRNAs targeting groups of paralogs encoding transcription factors by Jover-Gil and collaborators (Jover-Gil et al. 2014). In this case, 338 amiRNA-expressing Arabidopsis lines were generated, each of which expressed an amiRNA designed to simultaneously inactivate a set of two to six paralogous transcription factors. This collection was used to identify 21 amiRNAs causing vegetative leaf morphological phenotypes (Jover-Gil et al. 2014).

In the previous examples, amiRNA-expressing lines were obtained by introducing an amiRNA transgene into the plant genome. However, besides the standard expression of amiRNAs in plants through transgenes, *aMIRNA* precursors have also been successfully expressed from several plant DNA viruses through the so-called MIR-VIGS approach. Because viruses move throughout the plant, amiRNAs were also expressed systemically and silencing effects were visible in all those tissues the virus could invade. In all cases, plant DNA viruses used in MIR-VIGS belong to the genus Begomovirus, family Geminiviridae, and include *Cabbage leaf curl virus* (CaLCuV) (Tang et al. 2010), *Cotton leaf crumple virus* (CLCrV) (Gu et al. 2014), and the viral satellite DNA vector of *Tomato yellow leaf curl China virus* (TYLCCNV) (Ju et al. 2017). Although these vectors have been used mainly to silence endogenous reporter genes, they may constitute a useful tool for functional genomics in plants.

Finally, a recent report by Zhang and colleagues has offered new improvements in the amiRNA technology aimed to increase the levels of amiRNA-induced silencing (Zhang et al. 2018a). First, the authors developed a system in which the amiRNA was embedded into a portable intron within a fluorescent reporter. The basis of this system is that both the fluorescent reporter and amiRNA are produced from the same transcript, and thus the fluorescent reporter serves as a visible surrogate for checking amiRNA efficacy in vivo. And second, efficient multiplexing of several amiRNAs in the same construct was achieved by adding various amiRNA precursors in tandem, each of which was flanked by tRNA-processing sites.

1.4.1.2 Silencing of Non-coding Genes

AmiRNAs have also been used to silence endogenous *MIRNA* genes, to study the function of new miRNAs or to differentiate the function of individual members of a *MIRNA* family. Eamens and co-workers first reported the use of amiRNAs in Arabidopsis to target one or multiple miRNA family members, by targeting the mature miRNA or precursor stem–loop sequence, respectively (Eamens et al. 2011). Interestingly, these results suggest that sRNA-guided cleavage function could occur not only in the cytoplasm but also in the nucleus, thus providing new insights in the mechanisms of sRNA-mediated silencing. In another study, also in Arabidopsis, silencing of endogenous *MIR408* by amiRNAs caused impaired plant growth and

highlighted the importance of miR408 accumulation level for proper plant vegetative development (Zhang and Li 2013).

1.4.2 Artificial/Synthetic tasiRNAs

AtasiRNAs/syn-tasiRNAs have been used to study gene function and improve crops (Zhang 2014). They were first employed a decade ago to study the biogenesis of tasiRNAs from *TAS* transcripts in Arabidopsis (de la Luz Gutierrez-Nava et al. 2008). In most cases, expressed atasi/syn-tasiRNAs targeted genes with visible loss-of-function phenotypes such as *PHYTOENE DESATURASE* (*PDS*), *GREEN FLUORESCENT PROTEIN* (*GFP*), or *CHLORINA42* (*CH42*). Seminal findings included the observation that (i) AGO1-miR173 complexes initiate phased siRNA formation in plants (Montgomery et al. 2008b), (ii) miR390 associates exclusively with AGO7, (iii) miR390-AGO7 complexes function in distinct cleavage or non-cleavage modes at two target sites in *TAS3a* transcripts (Montgomery et al. 2008a), and (iv) tasiRNAs have a greater range in cell nonautonomous movement compared to miRNAs (de Felippes et al. 2011). A summary of representative examples of use of atasi/syn-tasiRNA in gene function is shown in Table 1.3.

More recently, atasi/syn-tasiRNA tools have been used to confer enhanced antiviral resistance (Chen et al. 2016; Carbonell and Daros 2017; Carbonell et al. 2019b), because the multitargeting of viral RNAs with multiple atasi/syn-tasiRNAs from a single construct limits virus ability to mutate target sites and escape the resistance (Carbonell et al. 2016, 2019a).

1.5 Concluding Remarks and Future Perspectives

Still in the genome editing era, art-sRNA-based RNAi tools offer a variety of advantages for functional genomic studies in order to dissect the function of any desired gene or gene network. Art-sRNAs (i) are highly specific, (ii) allow the functional study of genes whose complete knock-out is lethal, (iii) allow the study of genes in a spatio-temporal manner, as target silencing can be induced at specific times and/or at specific places by using inducible and/or tissue-specific promoters, respectively, (iv) should allow the fine-tuned regulation of target transcript levels to generate an allelic series for a knock-down gene, (v) can target duplicated genes (and gene families), antisense transcripts or individual isoforms, and (vi) can be multiplexed in single constructs for multisilencing. Moreover, the development of high-throughput methodologies to generate art-sRNA constructs should definitely facilitate the use of art-sRNA-based tools not only in gene function studies but also in obtaining next generation crops.

Table 1.3 Examples of uses of artificial/synthetic tasiRNAs to study gene function in plants

Plant species	Target(s)[a]	Gene function studied	References
Arabidopsis thaliana	*Ath-CH42*	Movement of silencing signal	de Felippes et al. (2011)
		TAS1a-derived tasiRNA biogenesis	Felippes and Weigel (2009)
		TAS3-derived tasiRNA biogenesis	de Felippes et al. (2017)
	Ath-FAD2	*TAS1c*-derived tasiRNA biogenesis	de la Luz Gutierrez-Nava et al. (2008)
	Ath-FT	Modulation of flowering time	López-Dolz et al. (2020)
	Ath-PDS	*TAS1c*-derived tasiRNA biogenesis	Montgomery et al. (2008b)
		TAS2-derived tasiRNA biogenesis	Yoshikawa et al. (2013)
		TAS3-derived tasiRNA biogenesis	Montgomery et al. (2008a)
Nicotiana benthamiana	*Ath-PDS*	*TAS1c*-derived tasiRNA biogenesis	Montgomery et al. (2008b)
		AGO7 miRNA loading; *TAS3a*-derived tasiRNA biogenesis	Montgomery et al. (2008a)
	Cme-ARF3	Identification of melon *TAS3* locus	Cervera-Seco et al. (2019)
	GFP	*TAS1c*-derived tasiRNA biogenesis	Montgomery et al. (2008b)
	Nbe-SU	Chlorophyll synthesis	López-Dolz et al. (2020)

[a]*ARF3, AUXIN RESPONSE FACTOR3; CH42, CHLORINA42; FAD2, FATTY ACID DESATURASE2; PDS, PHYTOENE DESATURASE; SU, SULPHUR*

Acknowledgements This work was supported by grants from Ministerio de Ciencia, Innovación y Universidades (MCIU, Spain), Agencia Estatal de Investigación (AEI, Spain), and Fondo Europeo de Desarrollo Regional (FEDER, European Union) [RTI2018-095118-A-100 and RYC-2017-21648 to A.C., and PRE2019-088439 to A.E.C.], and from Consejo Superior de Investigaciones Científicas (CSIC, Spain) [JAEINT_20_01312 to T.M.G].

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Chapter 2
Recent Advancements in MIGS Toward Gene Silencing Studies in Plants

Debee Prasad Sahoo

Abstract In plants, RNA interference (RNAi) causes gene silencing in which small RNAs (sRNAs) inhibit gene expression by causing sequence-specific degradation of target transcripts. Several RNAi-based tools have been developed and optimized to study gene function and trait improvements in plants. One recent strategy based on miRNA-triggered secondary small interfering RNAs (siRNAs) through trans-acting siRNA (tasiRNA) pathway has been developed for efficient gene silencing. In plants, miRNA-mediated cleavage of noncoding *TAS* RNAs triggers production of tasiRNAs which cause downregulation of one or more target genes. MiRNA-induced gene silencing (MIGS) works on this module in which a single miRNA target site fused with a target gene fragment in a vector triggers production of tasiRNAs and subsequent target gene silencing in plant cells. This technology has been successfully employed to silence one or more target genes to study their role in plant development and stress response. It has gained much attention due to its ease of design and capacity to silence multiple paralogous genes simultaneously. Further, MIGS vector designing does not require whole genome information, making it suitable to be used in plant species which lacks this information. This chapter summarizes recent progress in MIGS and its application in gene function studies and trait improvements.

Keywords miRNA · MIGS · Gene silencing · tasiRNAs · phasiRNAs

2.1 Introduction

Since the discovery of plant small RNAs (sRNAs), RNA is at the center of plant functional genomics studies (Morris and Mattick 2014) and has paved the way for exploitation of sRNAs in deciphering gene function via gene silencing. In plants, sRNAs are produced from double-stranded RNA precursors by Dicer-like (DCL) enzymes (Axtell 2013). Plant sRNAs are mainly categorized into microRNAs (miRNAs) and small interfering RNAs (siRNAs). The former gets excised from

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G. Tang et al. (eds.), *RNA-Based Technologies for Functional Genomics in Plants*,
Concepts and Strategies in Plant Sciences, https://doi.org/10.1007/978-3-030-64994-4_2

partially double-stranded regions of hairpin transcripts, whereas siRNAs originate from perfectly complementary double-stranded RNAs (Yoshikawa 2013). After the formation of miRNA and siRNA duplex, one strand of each duplex is loaded onto an Argonaute (AGO) protein and a RNA-induced silencing complex (RISC) is formed in association with other protein factors. These RISCs are guided by the AGO-bound sRNAs to target complementary sequences and regulate target gene expression either by transcriptional silencing and/or translational inhibition or degradation of transcript (Wei et al. 2012; Voinnet 2009; Eamens et al. 2008).

In addition to this, miRNA and siRNA-directed cleavages produce sequence templates to generate double-stranded RNA (dsRNA) by the action of RNA-dependent RNA polymerases (RDRs), which are further processed by DCL enzymes to generate secondary siRNAs (Schwab and Voinnet 2010; Baulcombe 2007; Voinnet 2008). Some secondary siRNAs, generated from the end of dsRNA, originate from an AGO-catalyzed cleaved RNA at a miRNA target site are 21-nt phased siRNAs (phasiRNAs) (Axtell 2013; Rajeswaran et al. 2012). PhasiRNAs which are able to repress different target loci other than the loci of their origin are known as trans-acting siRNAs (tasiRNAs) (Axtell 2013).

In Arabidopsis, four gene families encoding trans-acting siRNA (tasiRNA) precursors (TAS) have been identified (Fei et al. 2013). TAS1, TAS2, and TAS4 precursors are targeted by 22-nt asymmetric miRNAs/AGO1 complex by one-hit mechanism to generate tasiRNAs in Arabidopsis and related species, whereas TAS3 precursors generate tasiRNAs triggered by 21-nt symmetric miR390/AGO7 complex by two-hit mechanism (Cuperus et al. 2011; Axtell et al. 2006).

Based on miRNA-triggered secondary siRNA biogenesis, three different classes of silencing tools have been developed—(i) artificial synthetic tasiRNA (atasiRNA/syn-tasiRNA), (ii) miRNA-induced gene silencing (MIGS), and (iii) artificial miRNA (amiRNA). These tools have been extensively used to induce gene silencing in plants (Carbonell 2019). MIGS has an advantage over other techniques due to its ease of design (de Felippes et al. 2012; de Felippes 2013) and capacity to silence multiple genes, simultaneously. Current chapter describes MIGS as an effective gene silencing technology and recent advancements in gene silencing studies in plants, employing this strategy.

2.2 Biogenesis of MicroRNA-Triggered Secondary siRNAs

The tasiRNA pathway involves two different mechanisms, named as the “one-hit” and “two-hit” models (Fei et al. 2013). In the one-hit model, a 22-nt asymmetric miRNA directs cleavage of a tasiRNA precursor transcript by AGO1 (Fig. 2.1). Then, RDR6 along with a suppressor of gene silencing 3 (SGS3) catalyzes to synthesize a complementary RNA strand to form dsRNA molecule from the 3’ end of the cleaved product. Finally, endonuclease DCL4 cleaves the dsRNA every 21 nt from the first cleavage point to generate a population of secondary siRNAs (Yoshikawa et al. 2005). It has been reported that very few 22-nt asymmetric miRNAs trigger

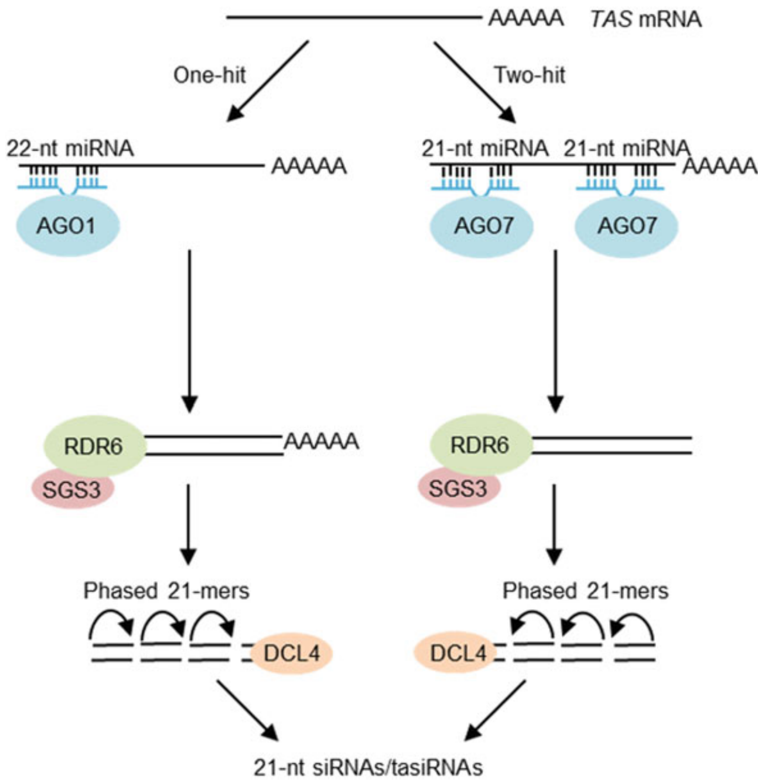

Fig. 2.1 Biogenesis of miRNA-triggered secondary siRNAs. In "one-hit" model, TAS transcript is targeted and cleaved by 22-nt miRNA/AGO1 complex whereas in "two-hit" model TAS transcript is targeted by two numbers of 21-nt miRNA/AGO7 complex and cleaved by miRNA/AGO7 complex closer to 3' end. Both the models recruit RDR6 and SGS3 for dsRNA synthesis and DCL4 for the production of 21-nt tasiRNAs

tasiRNAs production (Chen et al. 2010; Cuperus et al. 2010). In the two-hit model, the tasiRNA precursor transcript is targeted by two 21-nt miRNA390/AGO7 complexes (Fig. 2.1). The miRNA-AGO cleaves the target transcript closer to the 3' end whereas the other target site remains intact. Then RDR6 and SSG3 act to form dsRNA from the AGO-miRNA cleavage site to the site bound by other complex and finally DCL4 processes the dsRNA for 21-nt tasiRNAs that are phased with respect to the cleaved end (Axtell et al. 2006). The produced tasiRNAs target specific complementary RNAs in *trans* to degrade those targeted RNAs. Both one-hit and two-hit tasiRNA pathways have been exploited to design different constructs to knockdown target genes in plants.

2.3 Gene Silencing Technologies Based on tasiRNA Pathway

Based on tasiRNA pathway, atasiRNA/syn-tasiRNA approach was first developed to study gene silencing. In this approach, the endogenous tasiRNAs in a TAS precursor is substituted by a fragment containing one or more atasiRNAs/syn-tasiRNA sequences (Carbonell 2019). When these constructs are transferred to plants, engineered transcripts are cleaved by miRNA/AGO complex and one of the cleaved fragments is converted to dsRNA, which is processed further into 21-nt phased tasiRNAs. Then atasiRNA/syn-tasiRNA-guided strands are incorporated into AGO1 to induce silencing of one or more transcripts (Carbonell 2019). Several attempts have been made to efficiently knockdown one or multiple genes in gene function studies in Arabidopsis (de la Luz Gutiérrez-Nava et al. 2008; Montgomery et al. 2008a, b; Carbonell et al. 2014) by using this technology. The major advantage of this approach is a possibility of producing several atasiRNAs from one TAS precursor, which can target different target sequences at different locations (Carbonell et al. 2014). Another advantage of this technique is less chance of off-targeting. But, to design atasiRNA molecules to specifically downregulate certain genes, the entire genome information is necessary to minimize the chances of unwanted silencing (Ossowski et al. 2008).

The artificial miRNA (amiRNA) constructs are prepared by incorporating amiRNA and amiRNA* sequences in place of endogenous miRNA and miRNA* in a miRNA precursor. Upon transformation into plants, amiRNAs of the desired sequence get accumulated as a result of which the endogenous target transcripts would get silenced (Schwab et al. 2006). This approach has demonstrated high specificity and ability to silence multiple genes, however the constructs designing needs multiple steps of PCR to replace mature miRNA in a precursor backbone with amiRNA (Schwab et al. 2006; Ossowski et al. 2008). McHale et al. (2013) have successfully used amiRNA approach to knockdown *CHALCONE SYNTHASE* (CHS) in Arabidopsis.

Another approach based on tasiRNA pathway is termed as miRNA-induced gene silencing (MIGS), which was firstly used in Arabidopsis by expressing a target gene fused to an upstream miR173 target sequence (de Felippes et al. 2012). When MIGS constructs are introduced into plants, the miR173-mediated cleavage triggers the synthesis of secondary siRNAs which promote silencing of related target genes. MIGS can be used to knockdown multiple genes simultaneously by using a single vector by linking of different gene fragments, each with one miRNA target site (de Felippes et al. 2012). The major advantage of MIGS technology is that genome information is not required to design the constructs to be used in different plant species. But the risk of off-targeting is a major concern in employing this technology.

2.4 MicroRNA-Induced Gene Silencing (MIGS) and Its Advantages

Initially MIGS constructs were designed by taking miR173 target site followed by the target gene fused downstream of that (Fig. 2.2) (de Felippes et al. 2012). miR173, an asymmetric 22-nt miRNA, is able to trigger production of tasiRNAs from the fused transcript (Chen et al. 2010; Cuperus et al. 2010). The miR173/AGO1 complex guides the cleavage of the transcript which triggers RDR6-dependent synthesis of dsRNA and subsequent processing by DCL4 to release phased tasiRNAs. These tasiRNAs target the endogenous genes for efficient silencing. Since miR173 is absent in non-Arabidopsis species, so miR173 co-expression is required along with MIGS transgenes to induce tasiRNA production (de Felippes et al. 2012).

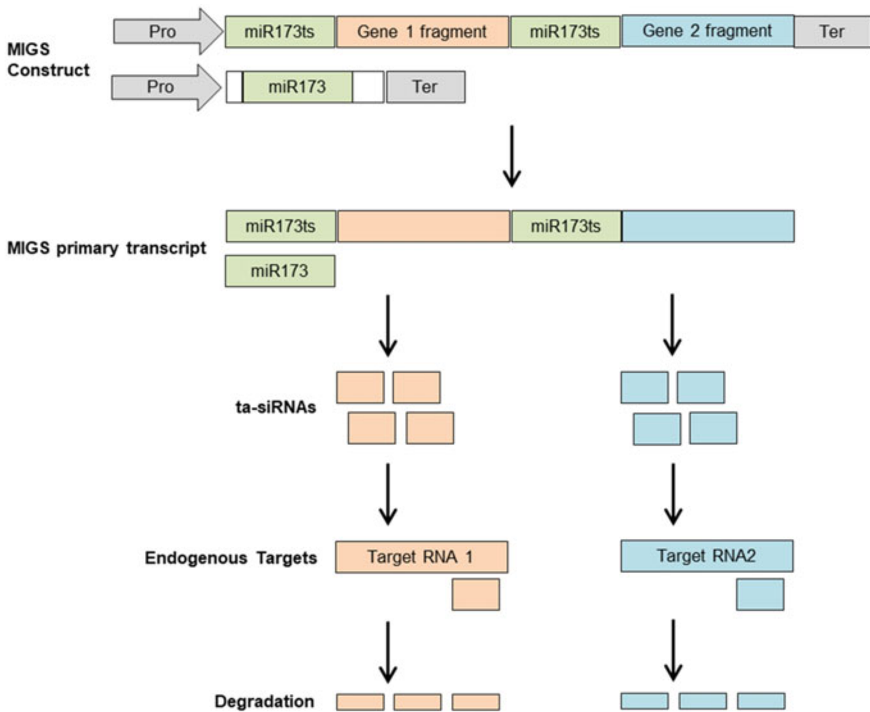

Fig. 2.2 A model MIGS construct and silencing mechanism. A MIGS construct can be prepared by placing a miRNA target sequence (e.g., miR173ts) in front of a target gene fragment. Multiple combinations can be used in a single cassette to downregulate multiple genes. Upon transformation into plant cells, it undergoes to form a long transcript having miR173ts and complementary transcript to each gene fragment. Binding of miR173 to the miR173ts triggers production of tasiRNAs and subsequent silencing of endogenous complementary genes by tasiRNAs. Pro (promoter), Ter (terminator)

Other 22-nt miRNAs like miR1514a.2 have also been reported to trigger tasiRNA production in soybean and the MIGS constructs have efficiently demonstrated to knockdown target genes in soybean (Jacobs et al. 2016). MiR390 which triggers production of tasiRNAs by two-hit model from TAS3 transcripts has also been used to prepare MIGS construct (Felippes and Weigel 2009). Since miR390 associates only with AGO7, miR390-based MIGS would be limited to AGO7 expression site, that is only in the vascular system (Montgomery et al. 2008b).

MIGS has major advantages over other technologies due to its easiness of design (de Felippes et al. 2012; de Felippes 2013). With one-step PCR, one target gene can be fused downstream of a miRNA target site which can trigger tasiRNA production. MIGS is very much effective in co-silencing multiple genes through a single vector construct by linking different target fragments, each with one miRNA target site, thus saving time. Sequence similarity between different target genes is not necessary since specific MIGS module is generated for each target separately (de Felippes et al. 2012). Since this technology does not require genome-wide data, it can be used in gene silencing studies of plant species which lacks this information (de Felippes 2019). The predictable pattern of produced siRNAs and specific expression profiles of miRNA triggers add extra level of control in MIGS compared to other gene silencing technologies (Jacobs et al. 2016).

2.5 Gene Silencing Studies in Plants Using MIGS

For the first time de Fellipes successfully employed MIGS to induce gene silencing of four Arabidopsis genes (*AGAMOUS*, *EARLY FLOWERING 3*, *FLOWERING LOCUS T*, *LEAFY*) in *A. thaliana* (de Felippes et al. 2012). Using MIGS technology Benstein et al. (2013) efficiently silenced *Arabidopsis* phosphoglycerate dehydrogenase1 (PGDH1) to study its role in Phosphoserine pathway, which has an important function in plant development. Using MIGS2.1 vector, silencing was successfully induced in C-terminally encoding protein1 (*CEP1*) gene in *Medicago trancatula,* which plays a major role in root development (Imin et al. 2013). To characterize the role of *Phosphoserine Aminotransferase1* (PSAT1) in serine biosynthesis pathway and its role in plant growth, Wulfert and Krueger (2018) used MIGS technology to downregulate *AtPSAT1* gene and obtained strong growth inhibition in both shoots and roots of *PSAT1*-silenced lines. Starch composition of rice was altered by silencing granular-bound starch synthase (GBSS) gene by using MIGS which effectively reduced amylose content in rice endosperm (Zheng et al. 2018). The authors also compared silencing efficiency of different MIGS constructs and found that the silencing efficiency was related to the selection of MIGS interfering target sites and specificity of the target genes. The target sites with high sequence homology found to be more efficient in interfering certain genes (Zheng et al. 2018). To study the role of microProteins in flowering behavior, Graeff et al. (2016) successfully reduced the expression levels of microProteins miP1a and miP1b using MIGS in *Arabidopsis thaliana.* In petunia, MIGS efficiently induced gene silencing of chalone synthase

(CHS) and phytone desaturase (PDS) and resulted in albino plants (Han et al. 2015). The authors carried out deep sequencing and concluded that processing of miRNA precursor in petunia is different from Arabidopsis. Zhao et al. (2015) demonstrated antiviral resistance in *Nicotiana benthamiana* targeting 3’ noncoding region or capsid protein-coding region of *Plum pox virus* (PPV) RNA by producing siRNAs through MIGS. MIGS was successfully employed to achieve viral resistance in tobacco and tomato by downregulating two RNAi suppressor proteins, AC2 and AC4, of geminivirus Tomato leaf curl New Delhi virus (ToLCNDV) (Singh et al. 2015). The authors used miR390-based MIGS vector to achieve silencing of target genes.

Jacobs et al. (2016) identified nine tasiRNA loci in soybean and experimentally validated corresponding targets by silencing a transgenic GFP gene and two endogenous genes by developing transgenic hairy roots and plants. The authors demonstrated the use of another 22nt-miRNA, miR1514 in constructing MIGS vectors. MIGS triggered by miR1514a.2 was successfully tested by silencing nodulation factor receptor kinase 1α (NFR) and putative cytochrome P450 CYP51G1 in soybean hairy roots and whole plants (Jacobs et al. 2016) (Table 2.1).

Table 2.1 Application of MIGS in gene silencing studies of model and crop plants

miRNA trigger	Plant species	Targets[a]	References
miR173	*Arabidopsis thaliana*	CH42	Felippes and Weigel (2009)
		AG, ELF3, FT, LFY	de Felippes et al. (2012)
		PGDH1	Benstein et al. (2013)
		miP1a, miP1b	Graeff et al. (2016)
		PSAT1	Wulfert and Krueger (2018)
	Medicago truncatula	CEP1	Imin et al. (2013)
	Nicotiana benthamiana	PPV	Zhao et al. (2015)
	Petunia hybrida	CHS, PDS	Han et al. (2015)
	Oryza sativa	GBSS, LAZY1, PDS, ROC5	Zheng et al. (2018)
miR390	*Arabidopsis thaliana*	CH42	Felippes and Weigel (2009)
	Nicotiana tabacum *Solanum lycopersicum*	ToLCNDV, ToLCGV	Singh et al. (2015)
miR1514a.2	*Glycine max*	NFR1α, P450 CYP51G1	Jacobs et al. (2016)

[a]Abbreviations: CH42, CHLORINA 42; AG, AGAMOUS; ELF3, EARLY FLOWERING 3; FT, FLOWERING LOCUS T; LFY, LEAFY; PGDH1, PHOSPHOGLYCERATE DEHYDROGENASE 1; miP1a, microProtein 1a; miP1b, microProtein 1b; PSAT1, PHOSPHOSERINE AMINOTRANSFERASE 1; CEP1, C-TERMINALLY ENCODED PEPTIDE 1; PPV, Plum pox virus; CHS, CHALCONE SYNTHASE; PDS, PHYTOENE DESATURASE; GBSS, GRANULE BOUND STARCH SYNTHASE 1; LAZY1, shoot gravitropism gene; ROC5, RICE OUTERMOST CELL-SPECIFIC 5; ToLCNDV, Tomato leaf curl New Delhi virus; ToLCGV, Tomato leaf curl Gujarat virus; NFR1α, NODULATION FACTOR KINASE 1α; P450 CYP51G1, putative cytochrome P450 CYP51G1

2.6 Limitations of MIGS and Steps to Overcome the Limitations

There is some risk of off-targeting associated with MIGS. All the population of siRNAs produced from MIGS construct is capable of silencing targeted sequences. There is a possibility that the non-intended targets which share sequence similarities may get silenced (de Felippes 2019). And also, few tasiRNAs derived from MIGS load into AGO1 and others either load to different AGOs or get degraded (Carbonell 2019).

In case of MIGS, the tasiRNAs form in a phased manner due to miRNA-triggered cleavage and are highly predictable (Allen et al. 2005; Montgomery et al. 2008b; de Felippes 2013; Felippes and Weigel 2009). The formation of phased tasiRNAs can be predicted by bioinformatics softwares like pssRNAMINER, tasiRNAdb, and SoMART, which could be used for minimizing off-target gene silencing (Pandey et al. 2015). The use of endogenous miRNAs in MIGS vector construction is also useful to minimize the off-target effects of exogenous miRNA expression (Jacobs et al. 2016).

Like other silencing tools, a variety of siRNAs are produced from the template dsRNA. To choose gene specificity in MIGS, the gene fragments selected, should share little sequence similarity and in that case selecting untranslated regions (UTRs), among related homologs, is a better choice (Wesley et al. 2001). Also, small gene fragments will be ideal for MIGS, ensuring predictability of phased tasiRNAs (Montgomery et al. 2008b; Felippes and Weigel 2009).

2.7 Conclusions

Nowadays genome editing like CRISPR/Cas9 has been widely used to mutagenize or edit gene sequence in plants (Belhaj et al. 2015; Rinaldo and Ayliffe 2015) to generate gene knock-out lines. But gene silencing technologies, used for gene function studies, are much simpler to use. Complete knock-out of a gene, as in the case of CRISPR/Cas9, may be lethal to plants, and cannot be recovered, but gene silencing technologies allow incomplete gene knockdowns to study the function of a gene. This technology allows tissue-specific gene silencing and also silencing of multiple genes. Furthermore, the technologies can be improved by overcoming the limitations and possible applications in plant functional genomics studies. With the advancement of plant genomics, comparative studies can be performed and with the availability of different computer programs, potential effective siRNAs and dsRNAs can be designed and analyzed to minimize off-target effects (Naito and Ui-Tei 2012; Naito et al. 2005).

MIGS is a miRNA-mediated RNA interference technology which has emerged recently. It has gained importance due to its simple construction steps, high specificity and efficiency in gene silencing. The ability to effectively silence multiple unrelated

genes using a single vector is an added advantage. Still it needs to be refined in terms to reduce off-targeting of the genes. The role and molecular mechanisms of recently identified phasiRNAs in different plant species needs to be explored. A deeper understanding of miRNA-triggered tasiRNA biogenesis, mode of action, and targeting efficacy is needed for better use of this technology in gene silencing studies and crop improvement.

Acknowledgements The author acknowledges the support of Science & Engineering Research Board (SERB), Government of India for National Post-Doctoral Fellowship (PDF/2016/001092) at Institute of Life Sciences, Bhubaneswar.

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Chapter 3
Target Mimic and Short Tandem Target Mimic Technologies for Deciphering Functions of miRNAs in Plants

Jun Yan, Jie Li, and Hengyan Zou

Abstract MicroRNAs (miRNAs) are central players in the regulation of gene expression at post-transcriptional level and are involved in numerous biological processes in both plants and animals. However, deciphering the function of a miRNA family, based on generation and characterization of miRNA mutants, has been difficult because of their small sizes and multiple functionally redundant members. The recently developed approaches termed Target Mimic (TM) and Short Tandem Target Mimic (STTM) overcome these barriers and can destroy specific miRNA function effectively. These methods have become a key tool in investigating miRNAs functions. In this chapter, we will introduce the development of TM and STTM, and the construction of STTM and its application in plants.

Keywords miRNA · Small RNA · Target mimic · Short tandem target mimic

3.1 Introduction

MicroRNAs (miRNAs) are small regulatory non-coding RNA molecules that play important roles in various biological processes (Ghildiyal and Zamore 2009). Plant miRNAs bind to target sites and negatively regulate gene expression by directing target mRNAs to undergo cleavage or translational inhibition (Baulcombe 2004). Since the first plant miRNA was discovered, more than 7000 mature miRNAs have been identified in different plant species in the past twenty years. The number of plant miRNAs is still expanding, with the help of high-throughput deep sequencing techniques (Kozomara and Griffiths-Jones 2011). Studies on the function of miRNAs have improved our understanding of their essential roles in gene regulatory networks. However, owing to their small size and functional redundancy, functional analysis of specific miRNA families has relied on the generation of transgenic lines expressing miRNA-resistant target genes or overexpressing miRNA encoding genes

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G. Tang et al. (eds.), *RNA-Based Technologies for Functional Genomics in Plants*,
Concepts and Strategies in Plant Sciences, https://doi.org/10.1007/978-3-030-64994-4_3

rather than the generation of miRNA genetic mutants (Baker et al. 2005; Mallory et al. 2005; Terentyev et al. 2009; Zhu et al. 2009; Zhang et al. 2011). Because miRNA usually modulates several target genes, using such approaches may produce misleading results. The ideal approach for deciphering miRNA function would be simultaneously silencing all members of a miRNA family. Invention of such tools will greatly facilitate the miRNA research field. In this chapter, we will introduce the tools commonly used to silence miRNAs-Target Mimic (TM) and Short Tandem Target Mimic (STTM) technologies, with a focus on STTM (Fig. 3.1; Table 3.1).

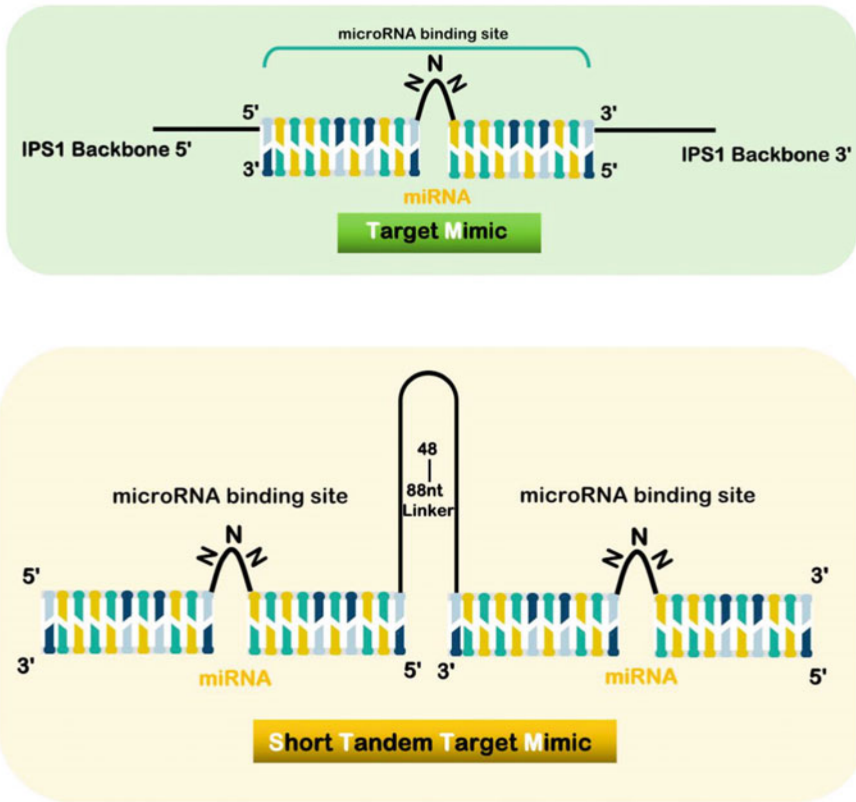

Fig. 3.1 Comparison of TM and STTM structures. A TM construct consists of one target mimic and STTM consists of two target mimics separated by a spacer of 48–88 nt

Table 3.1 A summary of STTM, along with examples, to investigate miRNA functions in plants

Targeted miRNA(s)	Species	References
miR399	Citrus	Wang et al. (2020)
miR319a	*Populus tomentosa*	Fan et al. (2020)
miR1917	Tomato	Yang et al. (2020)
miR6443	*Populus tomentosa*	Fan et al. (2020)
Nbe-miR1919c-5p	Tobacco	Du et al. (2020)
miR1150.3, miR1166.1	*Chlamydomonas reinhardtii*	Sun et al. (2020)
miR165/166	Tomato	Zhao et al. (2020)
miR861	Arabidopsis	Zhu et al. (2020)
miR171, miR390	Wild tomato (*S. habrochaites)*; *Arabidopsis*	Hou et al. (2019)
miR1916	Tomato	Chen et al. (2019)
milR-1, milR-7 AF-milR-4, AF-milR-7	*Metarhizium robertsii* *Aspergillus flavus*	Wang et al. (2019)
miR160, miR165/166, double mutant	Arabidopsis	Yang et al. (2019)
miR482/2118 family	Tomato	Canto-Pastor et al. (2019)
miR171	Tomato	Kravchik et al. (2019)
miR143-5p	Melanocyte	Qi et al. (2019)
miR164d, miR396b	Cucumber	Wang et al. (2019)
miR160, miR164, miR166, miR167, miR169, miR319, miR396, miR398, miR444, miR7695	Rice	Li et al. (2019)
miR390	Poplar	He et al. (2018)
miR1507a, miR1507c, miR482a, miR168a, miR1515a	Soybean	Bao et al. (2018)
miR166	Rice	Zhang et al. (2018)
miR508	Alpaca	Liu et al. (2018)
miR482b	Tomato	Jiang et al. (2018)
miR472a	Poplar	Su et al. (2018)
miR396	Medicago	Proust et al. (2018)
miR9678	Wheat	Guo et al. (2018)
miR156/157, miR160, miR165/166, miR167, miR171, miR319, miR159, miR319/159, miR172	Arabidopsis, tomato, rice, and maize	Peng et al. (2018) Teotia and Tang (2017)
miR1514a	Bean	Sosa-Valencia et al. (2017)
miR159	Rice	Zhao et al. (2017)

(continued)

Table 3.1 (continued)

Targeted miRNA(s)	Species	References
35 families including: miR398, miR172, miR156, miR166, miR159, miR160, miR171, miR441, miR1428	Rice	Zhang et al. (2017a)
Md-156ab, Md-395	Apple	Zhang et al. (2017b)
miR166	Rice	Zhang et al. (2017a), Teotia et al. (2017)
miR396	Tomato	Cao et al. (2016)
miR165/166, miR159	Tobacoo	Zhao et al. (2016)
miR160	Tomato	Damodharan et al. (2016)
miR160	Soybean	Nizampatnam et al. (2015)
miR165/166	Arabidopsis and tomato	Jia et al. (2015)
miR172, miR319; miR165/166	Tobacco and tomato	Sha et al. (2014)
miR165/166	Cotton	Gu et al. (2014)
miR9863	Barley	Liu et al. (2014a, b)

3.2 TM and STTM

The discovery of an endogenous mechanism termed target mimic (TM) paved the way for the invention of new tools to inhibit miRNA activity for further exploring miRNA function. This regulatory mechanism is used by the non-protein-coding gene *INDUCED BY PHOSPHATE STARVATION 1* (*IPS1*) to regulate miR399 activity in *Arabidopsis thaliana* (Franco-Zorrilla et al. 2007). miR399 is complementary to its target *PHOSPHATE 2* (*PHO2*), but partially complementary to *IPS1*, forming a central three-nucleotide bulge in the miR399/IPS1 duplex. This bulge can effectively prevent the cleavage of *IPS1* RNA by miR399. Under phosphate starvation conditions, both miR399 and *IPS1* are induced, and *IPS1* can sequester miR399 and reduce its ability to modulate *PHO2*. In this way, the miR399 mediated regulation of *PHO2* is impacted by *IPS1* in response to phosphate starvation. Based on the *IPS1*-derived structure, TMs have been designed to sequester miRNA families in *Arabidopsis* (Todesco et al. 2010). These TMs are about 500 nt in length and contain three central mismatches in the miRNA binding site (Fig. 3.1). TM technology has been used by many researchers to uncover miRNA functions.

Short tandem target mimic (STTM) was developed based on TM and has advantages over the *IPS1*-based method in uncovering miRNA function (Yan et al. 2012). The STTM sequence contains two non-cleavable miRNA binding sites, which can be used to target one specific miRNA or two different miRNAs. These two non-cleaveable miRNA binding sites are linked by a spacer of 48–88 nt. STTM can knockdown the expression of miRNA, likely through the induction of degradation of miRNAs. STTM technology has been widely applied in different model plants

and crops to reveal the important roles of miRNAs in regulating key agronomic traits (Zhang et al. 2017a; Peng et al. 2018).

The TM is about 500 nt in length and contains three central mismatches in the single miRNA binding site. The STTM has two non-cleavable miRNA binding sites and a spacer of 48–88 nt.

3.3 Construction of STTM

3.3.1 Design of STTM Structure

The STTM construct has two copies of miRNA binding sites. Three additional nucleotides (CTA) were introduced into each miRNA binding site between the 10th and 11th nucleotides from the 5′ end of the mature miRNAs. To target miRNAs, the two non-cleavable miRNA binding sites were designed based on sequence comparison. The two tandem miRNA binding sites can be identical or slightly different to target the members of the same miRNA family. The two miRNA binding sites can also be different to target members of two distinct miRNA families with different or partially conserved mature miRNA sequences. Currently, due to the low efficiency of STTM-triggered miRNA degradation with only one miRNA binding site, the STTM technology is not recommended to target two distinct miRNAs from different miRNA families. In some cases, the CTA introduced in the miRNA binding site is coincidently complementary to the TAG sequence after the 10th nucleotide in the target miRNA. In that case, to prevent the cleavage of miRNA binding site, a different trinucleotide should be used to replace CTA. Between the two miRNA binding sites, a relatively AT-rich spacer should be introduced to link the binding sites. The spacer is 48–88 nt in length and able to form a stem-loop structure, which makes the STTM more stable.

3.3.2 Promoter Selection

STTM structure can be driven by a variety of promoters. The choice of promoters depends on the researcher's particular purpose. For constitutive expression, a viral-origin constitutive promoter, such as the cauliflower mosaic (CaMV) 35S promoter, is commonly used. Some plant-origin constitutive promoters, such as the rice actin promoter and maize ubiquitin promoter, are also routinely used. The STTM structure can also be linked to inducible promoters that are activated by environmental conditions, chemicals, and hormones. The use of a tissue-specific promoter can allow the expression of STTM in a specific tissue or specific developmental stage of plant species. Endosperm-specific promoter has been used to express STTMs in rice seeds (Peng et al. 2018).

3.3.3 Plasmid Construction

A pOT2-Poly-Cis plasmid that contains a 2X35S promoter (d35S), 35S terminator (T-35S), and a screening marker gene was used as a template for PCR amplification (Yan et al. 2012; Tang et al. 2012). Primers designed for the generation of the pOT2-STTM were composed of a 3′ part with full base pairing to bind to the vector to initiate PCR extension, and a 5′ part that contained half of the STTM sequence (Fig. 3.2a). A

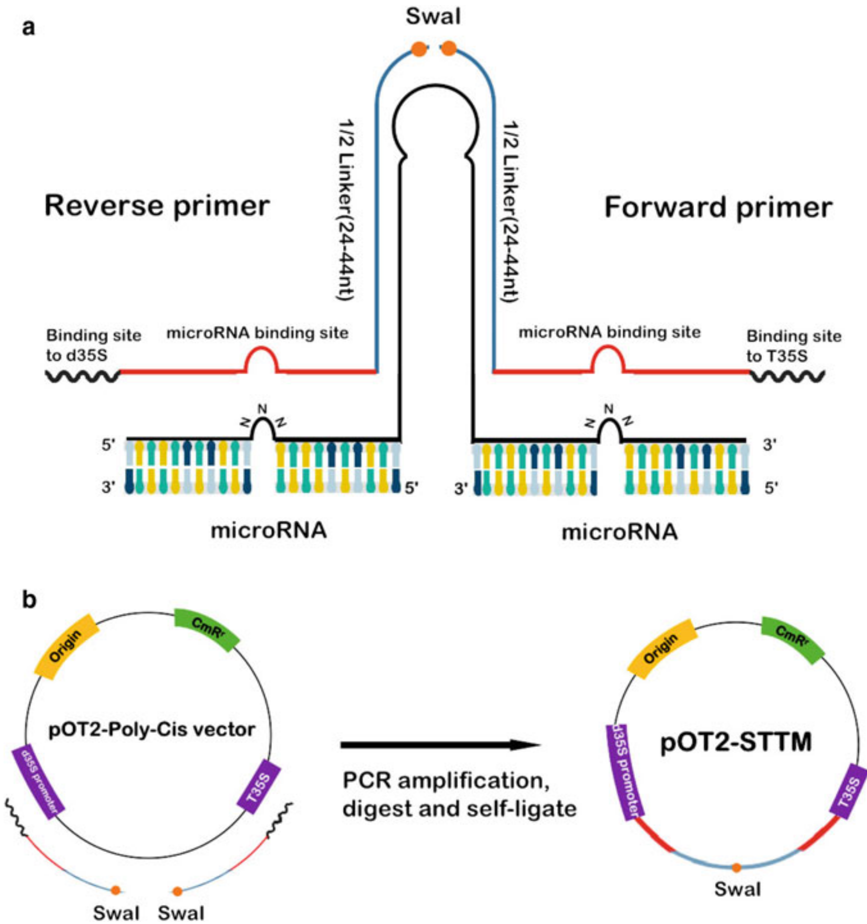

Fig. 3.2 Construction of an STTM construct. **a** STTM transcript structure and the design of STTM primers. For the STTM structure, the two miRNA binding sites are linked by 48–88 nt spacer. For the STTM primers, blue indicates the 5′portion of the primer, which is complementary to the stem region of the STTM structure; red indicates the middle portion of the primer, which is complementary to the miRNA binding sites of the STTM structure; black indicates the 3′ portion of the primer, which is complementary to the pOT2-Poly-Cis vector. **b** STTM PCR construction strategy. This strategy mainly contains PCR amplification, digestion, and subsequent ligation steps

SwaI site protected by a three-nucleotide GCC was introduced at the far 5′ end of the primer. The linear PCR products were digested with SwaI enzyme and then subjected to self-ligation to generate the pOT2-STTM (Fig. 3.2b), which was used as a template for subsequent PCR amplification. A pair of origin deletion primers, which contain a PacI site at the far 5′ end, was used for this PCR. The linear PCR products were then subjected to PacI digestion and subcloned into the modified binary vectors, such as pCambia1300-PacI, pCambia2300-PacI, and pFGC5941-PacI, all of which contain a PacI site(**A**).

3.4 Application of STTM

By applying the STTM strategy to study miRNA function, STTMs can be expressed in plant cells via different approaches, including stable transformation, virus-induced gene silencing (Sha et al. 2014), and *Agrobacterium*-mediated transient expression (Zhang et al. 2017b). The STTM approach has been previously applied to down-regulate mature miRNA expression in several species, including model plants and crops, and has proven to be an effective and powerful approach to investigate miRNA function. For example, in *Arabidopsis*, various miRNA families have been successfully inactivated using STTM (Yan et al. 2012; Peng et al. 2018), thus generating a useful resource for investigating the novel functions of miRNAs. Furthermore, in rice, compared with the wild-type line, the expression levels of target miRNAs, such as miR156, miR159, miR160, miR166, miR171, miR172, and miR398, can be downregulated up to 100-fold in transgenic lines (Zhang et al. 2017a), whereas those of miR156 can be downregulated approximately two-fold in the transgenic lines containing MIM constructs (Wang et al. 2015). Silencing rice miRNAs using STTM has uncovered both conserved and novel functions. Transgenerational stability is critical for functional studies and crop improvement. The observed phenotypes of transgenic STTM lines in *Arabidopsis* and rice have been demonstrated to be very stable across generations (Yan et al. 2012; Zhang et al. 2017a). This property will greatly facilitate future agronomic improvement. In addition to *Arabidopsis* and rice, STTM has also been used to inactivate different miRNA families in other species. For example, in tomato, silencing of miR482b using STTM revealed the functional response of miR482b to *Phytophthora infestans* infection (Jiang et al. 2018). Furthermore, inactivation of wheat-specific miR9678 provided evidence that miR9678 is required for seed germination (Guo et al. 2018). Knockdown of miR393 expression by STTM provided insight into the function of miR393 in soybean defense against *Phytophthora sojae* (Wong et al. 2014). A study on the silenced transgenic lines of STTM472a revealed the role of miR472a in plant immunity in *Populus trichocarpa* (Su et al. 2018). The STTM approach has also been used to silence miRNAs in *Medicago truncatula* transgenic roots (Proust et al. 2018). Thus, STTM is a great RNA-based technology and is essential for functional genomics in plants.

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Chapter 4
Silencing and Expressing MicroRNAs in Plants Through Virus-Based Vectors

Aihua Sha

Abstract The traditional methods for functional analysis of microRNAs is to create transgenic plants with either overexpression or loss of function of microRNAs. Expression vectors based on plant viruses have the advantages of easy infection, short time to get results, and high throughput nature. Here, we describe the approaches to discover microRNAs functions based on viral vectors in plants. We describe here the use of viral system as a tool for gene function analysis, strategies for functional analysis of microRNAs, plant viral vectors used for gene silencing or overexpression, and virus-based miRNA silencing or overexpression in plants. Finally, the perspectives of viral vectors in functional analysis of microRNAs and crop breeding are discussed.

Keywords Viral vectors, microRNAs · Mimic, short tandem target mimic, sponge

4.1 Introduction

Plant virus expression vectors can either overexpress or suppress gene expression in plants. Plant viral vectors can efficiently express the heterologous proteins when the gene is engineered into the viral genome. The heterologous proteins will be produced in significant quantities as virus replicates in the host. The protein of interest is expressed under a strong (duplicated) viral promoter such as the coat protein (CP) subgenomic promoter. The gene of interest is delivered to plant cells either as infectious nucleic acid copies of the vector or by *Agrobacterium*-mediated infection (Gleba et al. 2007).

Viral vectors can be also be used to suppress gene expression. A RNA-mediated defense mechanism, namely, post-transcriptional gene silencing (PTGS), where

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G. Tang et al. (eds.), *RNA-Based Technologies for Functional Genomics in Plants*,
Concepts and Strategies in Plant Sciences, https://doi.org/10.1007/978-3-030-64994-4_4

plants detect viral RNAs and initiate the PTGS-like response to degrade the foreign viral RNAs, exists. The specificity of PTGS depends on the activity of endogenous RNA-dependent RNA polymerase, which can produce short complementary RNA (cRNA) molecules by using specific RNAs as templates. The complementary sequences are targeted by these cRNAs which serve as a guide for RNA-degrading enzyme activity (Lindbo et al. 2001). The RNA-mediated defense is triggered by the viral vectors carrying host-derived sequence inserts, which target both the viral genome and the host gene corresponding to the insert. As a result, the symptoms appear in the infected plant due to loss of function or reduced-expression of the host gene (Ratcliff et al. 2001).

4.2 Strategies for Functional Analysis of MicroRNAs

MicroRNAs (miRNAs) are a class of small non-coding RNAs (ncRNAs) functioning as key regulators of gene expression that control multiple functions in plants and animals (Banks et al. 2012). In plants, the long ncRNA precursors containing a stem-loop secondary structure is processed to the mature miRNAs by an RNase III-like enzyme, DCL1, which coordinates with DRB1 (HYL1) and SE (Banks et al. 2012). One strand of the miRNA duplex, the guide strand, is loaded onto the AGO1 protein of the RNA-induced silencing complex (RISC) to recognize the target mRNA. miRNA mediates target mRNA cleavage at the post-transcriptional level or induces translational inhibition in plants (Banks et al. 2012).

Two reciprocal reverse genetic strategies are traditionally adopted to investigate the function of a particular miRNA in the plant, either, enhancing miRNA activity or blocking miRNA function (Jones-Rhoades et al. 2006). The former is achieved by transgenic overexpression of the miRNA, and the latter by either altering the miRNA gene (Allen et al. 2007) or expressing a miRNA-resistant target (Zhao et al. 2007). Recently, miRNA decoys were developed to overcome the problems challenged by traditional transgenic methods due to most miRNAs containing multiple functionally redundant members (Wong and Millar 2019). The expression of miRNA decoys through transgenic approaches can sequester or inhibit targeted miRNAs, which generates a loss-of-function miRNA effect. Three miRNA decoys have been developed in plants, that is, miRNA MIMICs (MIM), SHORT TANDEM TARGET MIMICs (STTMs), and miRNA SPONGEs (SPs). MIMs were first miRNA decoys used in plants, which were derived from the endogenous *Arabidopsis* gene *INSENSITIVE TO PHOSPHATE STARVATION 1 (IPS1). IPS1* encodes mRNA containing a 23-nucleotide sequence that was partially complementary to miR399 with a three-nucleotide mismatch 1oop at the miRNA binding site of miR399. The loop inhibited the cleavage of *IPS1* mRNA, which caused the sequestration of miR399 from its endogenous targets as the miR399-RISC complex remained bound to the *IPS1* RNA. As a result, the expression of endogenous targets was not repressed. The *IPS1* transcript sequence has been modified to inhibit other miRNA families by

replacing the miR399 bulged target with sequences complementary to other miRNAs (Franco-Zorrilla et al. 2007).

STTMs are the second miRNA decoys, which are ~100 nt short artificial transcripts containing two MIM binding sites separated by 48 nt spacers. STTMs have better efficiency to inhibit miRNAs in comparison to MIM in *Arabidopsis* and other plant species (Yan et al. 2012; Zhang et al. 2017). miRNA SPONGEs SPs are synthetic sequences containing multiple miRNA binding sites separated by 4 nt spacers. They compete for miRNA binding and perturb the endogenous miRNA-target mRNA interaction (Reichel et al. 2015). SPs were firstly developed in animal systems (Ebert et al. 2007), and have been shown to inhibit miRNA activity in plants as well (Reichel et al. 2015).

4.3 Virus-Based Gene Silencing or Gene Overexpression in Plant

Virus-based gene silencing or expression has proven to be a powerful tool for characterizing the function of genes in plants. The advantage of the viral vector in function analysis lies in its speed and easy adaptation to high throughput systems (Baulcombe 1999). Initially, viral vectors were designed to overexpress genes to obtain proteins and metabolites, for instance, overexpression of phytoene synthase and capsanthin-capsorubin synthase gene from a *Capsicum species* in *Nicotiana benthamiana* plants (Lindbo et al. 2001). Viral vectors can also be used to study gene function by transient overexpression. The expression of the *FEN* gene by the *Potato virus X* (PVX) gene expression system resulted in sensitivity to fenthion in tomato (Rommens et al. 1995). Overexpression of *MADS*-box gene, *SlMADS-RIN*, and *SBP*-box gene, *SlSPL-CNR*, by a modified *PVX* vector was able to complement non-ripening phenotype of *Ripening inhibitor* (*rin*) mutant and *Colorless non-ripening* (*Cnr*) mutant in tomato fruit, respectively (Kong et al. 2013).

Recently, dozens of virus vectors have been developed as virus-induced gene silencing (VIGS) tools to analyze the loss of the gene function (Table 4.1). The VIGS vectors are derived from viral RNA as well as DNA. In the past decades, most of the viral vectors were mainly used in dicotyledon species such as tobacco, tomato, *Arabidopsis*, etc. (Table 4.1). The *tobacco rattle virus* (*TRV*)-based vector is the most common viral vector used in a wide range of plant species. The *apple latent spherical virus (ALSV)* vector has shown excellent adaption in several important crops such as apple, pear, tomato, legume, *Cucurbit* species. Several viral vectors such as *Brome Mosaic Virus (BMV)*, *Barley Stripe Mosaic Virus (BSMV)*, *Bean Pod Mottle Virus (BPMV)*, *Cucumber Mosaic Virus (CMV)*, *Rice Tungro Bacilliform Virus (RTBV)*, *Rice Tungro Bacilliform Virus (RTBV)*, *Foxtail Mosaic Virus (FoMV)* have been successfully used in monocots (Table 4.1). These vectors were used in important food crops such as wheat, rice, and maize. The endogenous gene can be silenced in different tissues such as leaf, root, flower, fruit, which is dependent upon the viral vectors with different efficacies.

Table 4.1 The developed VIGS vectors used for gene silencing in plants

Virus	Plant species applied	Silenced tissue	Reference
RNA virus			
Apple latent spherical virus (ALSV)	*Arabidopsis*, apple, cucurbit species, legume, pear, tobacco, tomato	leaf, seed	Huang et al. (2012)
Brome mosaic virus (BMV)	barley, maize, rice, *Tall fescue*, sorghum, tobacco	leaf, flower	Huang et al. (2012), Kumar et al. (2018)
Barley stripe mosaic virus (BSMV)	*Aegilops tauschii, Avena*, barley, *Brachypodium distachyum*, wheat	root, leaf	Huang et al. (2012), Tavakol (2017)
Bean pod mottle virus (BPMV)	soybean, wheat	root, leaf, shoot	Huang et al. (2012)
Chinese wheat mosaic virus (CWMV)	tobacco, wheat	leaf	Yang et al. (2018)
Cucumber mosaic virus (CMV)	maize, soybean	Leaf, seed	Huang et al. (2012), Wang et al. (2016)
Cymbidium mosaic virus (CymMV)	*Phalaenopsis orchids*	flower	Huang et al. (2012)
Foxtail mosaic virus (FoMV)	*foxtail millet*, maize, wheat	leaf	Liu et al. (2016), Mei et al. (2016)
Potato virus X (PVX)	tobacco	Leaf, root, tuber	Huang et al. (2012)
Potato virus A (PVA)	tobacco	leaf	Huang et al. (2012)
Pea early browning virus (PEBV)	*Lathyrus odorata*, *Medicago truncatula*	leaf, shoot, root, flower, pod	Huang et al. (2012)
Poplar mosaic virus (PopMV)	tobacco	leaf	Huang et al. (2012)
Plum pox virus (PPV)	tobacco	leaf	Huang et al. (2012)
Soybean yellow common mosaic virus (SYCMV)	soybean	leaf	Lim et al. (2015)
Sunn-hemp mosaic virus (SHMV)	*Medicago truncatula*, tobacco	leaf	Huang et al. (2012)
Tobacco mosaic virus (TMV)	Tobacco	leaf	Huang et al. (2012)
Tobacco necrosis virus A (TNV-A)	Tobacco	leaf	Huang et al. (2012)
Tomato bushy stunt virus (TBSV)	Tobacco	leaf	Huang et al. (2012)
Tobacco rattle virus (TRV)	*Arabidopsis*, *Aquilegia vulgaris*, cotton, opium, petunia, populus, rose, *Rauwolfia*, strawberry, *Thalictrum dioicum*, tobacco, tomatto	Leaf, shoot, root, flower, fruit, silique	Huang et al. (2012), Jia et al. (2013), Shen et al. (2015), Corbin et al. (2015), Corbin et al. (2017), Cheng et al. (2018)

(continued)

Table 4.1 (continued)

Virus	Plant species applied	Silenced tissue	Reference
Tobacco ring spot virus (TRSV)	*Arabidopsis*, cucurbits, legumes, tobacco	leaf, flower, fruit	Zhao et al. (2016)
Turnip yellow mosaic virus (TYMV)	*Arabidopsis*	leaf, shoot, flower, silique	Huang et al. (2012)
White clover mosaic virus (WCIMV)	pea	leave	Ido et al. (2012)
DNA virus			
Abutilon mosaic virus (AbMV)	tobacco	leaf	Huang et al. (2012)
African cassava mosaic virus (ACMV)	mannihot, tobacco	leaf, root	Huang et al. (2012), Lentz et al. (2018)
Beet curly top virus (BCTV)	spinach, tomato	leaf, flower	Huang et al. (2012)
Cabbage leaf curl virus (CaLCuV)	*Arabidopsis*, tobacco	leaf	Huang et al. (2012)
Cotton leaf crumple virus (CLCrV)	cotton	leaf, flower, boll	Huang et al. (2012)
East African cassava mosaic virus (EACMV-K201)	cassava	leaf	Beyene et al. (2017)
Grapevine virus A (GVA)	grape, tobacco	leaf	Huang et al. (2012)
Pepper huasteco yellow vein virus (PHYVV)	pepper	fruit	Huang et al. (2012)
Rice tungro bacilliform virus (RTBV)	rice	leaf	Huang et al. (2012), Kant and Dasgupta (2017)
Tomato golden mosaic virus (TGMV)	tobacco	leaf	Huang et al. (2012)
Tomato leaf curl virus (ToLCV)	tobacco, tomato	leaf	Huang et al. (2012)
RNA satellite virus Satellite of tobacco mosaic virus (STMV)	tabacum	Leaf, flower	Huang et al. (2012)
Tomato yellow leaf curl China virus (TYLCCNV)	petunia, tobacco, tomato	leaf, shoot, root, flower, fruit	Huang et al. (2012)
Tobacco curly shoot virus (TbCSV)	Tobacco	leaf	Huang et al. (2012)
TbCSV Alphasatellite	petunia, tobacco, tomato	leaf, flower	Huang et al. (2012)

4.4 Virus-Based miRNA Expression or Silencing in Plants

Plant miRNAs play critical roles in multiple biological processes, and traditional methods for functional analysis of miRNA require the generation of stable transgenic plants, which is time- and cost-consuming processes. Viral vector-based transient gene expression techniques can overcome those limitations because they do not require the generation of stable transgenic plants. Furthermore, transient expressions are also useful in characterizing lethal phenotypes (Sha et al. 2014). Viral vectors are also suitable for analyzing miRNA functions in plant species that are not readily amenable to genetic transformation. Using viral vectors, the artificial and/or native miRNAs can be overexpressed to specifically suppress their target genes, whereas MIMs, STTMs and/or SPs can be overexpressed to inhibit the activity of miRNAs in plants (Fig. 4.1).

Tang et al. first reported that the overexpression of an endogenous or artificial miRNAs using a *cabbage leaf curl virus (CaLCuV)*-based vector in plants (Tang et al. 2010). The expression of endogenous genes *PDS, Su, CLA1*, and *SGT1* were effectively silenced by the artificial miRNAs overexpressed by the *CaLCuV* vector in *Nicotiana benthamiana* (Tang et al. 2010). Meanwhile, the ectopic expression of endogenous miR156 and miR165 by the viral vector resulted in abnormal developmental phenotypes in *N. benthamiana* (Tang et al. 2010). Henceforth, the CLCrV (cotton leaf crumple virus) vector was developed to ectopically express endogenous miR156 in *G. hirsutum*, in which down regulation of miR156-targeted mRNAs caused abnormal leaf development phenotypes (Gu et al. 2014). Jian et al. demonstrated that the *BSMV* system successfully overexpressed the endogenous miR156 and an artificial miRNA (*amiR-PDS*) against phytoene desaturase gene *PDS* in wheat (Jian et al. 2017). Ju et al. showed that *TYLCCNV (Tomato yellow leaf curl China virus)* vector could overexpress both the artificial miRNAs and endogenous siRNAs in *Nicotiana benthamiana*. The endogenous genes *PDS*, *Su*, and *PCNA* were silenced

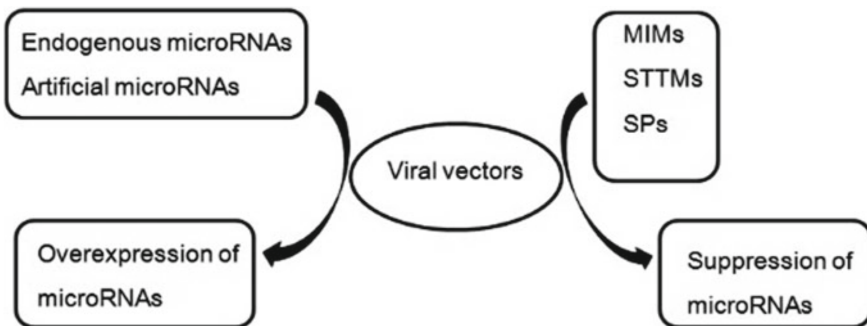

Fig. 4.1 The flow chart of overexpression or suppression of microRNAs in plants using viral vectors. For details of virus-based expression of endogenous or artificial miRNAs, refer to (Tang et al. 2010; Jian et al. 2017). For information of virus-based expression of MIMs and STTMs, refer to Sha et al. (2014), and for SPs, refer to Ebert et al. (2007)

by overexpressing the corresponding artificial miRNAs. Meanwhile, the endogenous miR156 and siRNA athTAS3a 5’D8(+) could also be overexpressed based on *TYLCCNV* system (Ju et al. 2017).

In addition to overexpress the artificial and/or native miRNAs, the viral vectors could be also applied to silence endogenous miRNAs in plants. Sha et al. modified the *TRV*-based VIGS vector to deliver the MIMs or STTMs of selected miRNAs in tobacco and tomato (Sha et al. 2014). They found that expression of MIMs or STTMs through *TRV* vector could silence the endogenous miR172, miR165/166, miR319 in *Nicotiana benthamiana* or tomato (*Solanum lycopersicum*), which caused developmental defects. Yan et al. also demonstrated that the *TRV*-based VbMS system worked well in *Arabidopsis* (Yan et al. 2014). They introduced the MIM of miR156, miR319, or miR164 into the viral genomic RNA, and found that the viral-inoculated plants and the stably transformed *Arabidopsis* plants showed the same phenotypes (Yan et al. 2014). Chen et al. has developed a protocol for MR VIGS (miRNA-based virus-induced gene silencing) in plants, based on two virus-based miRNA expression systems, *CaLCuV* and *TRV* vectors (Chen et al. 2015). Jiao et al. used modified *BSMV* system to suppress miR159a and miR3134a through the expression of MIM or STTM in wheat. The expressions of mature miR159a and miR3134a were decreased with a concomitant increase in the transcript levels of their target genes (Jiao et al. 2015). Jian et al. also successfully used *BSMV* to knock down the endogenous miR156 and miR166 levels in wheat (Jian et al. 2017). Zhao et al. showed that the *PVX*-based MIM expression could strongly silence miRNAs in *Nicotiana benthamiana* and potato (Zhao et al. 2016, 2020). Overexpression of STTMs against miR165/166 and miR159 by *PVX* led to defective phenotypes that were similar to those of transgenic plants (Zhao et al. 2016). Du et al. developed a *LS-CMV* (*Cucumber mosaic virus*) -based vector to express MIM of miR159. The depletion of miR159 in *Arabidopsis* resulted in symptoms similar to those of *Fny-CMV* infected plants, which disrupted miRNA-regulated development (Du et al. 2014). Yang et al. demonstrated that the *CWMV* (*Chinese wheat mosaic virus*) could be used to express the MIM in wheat to suppress miR165/166 and miR3134a (Yang et al. 2018). The *cucumber mosaic virus* strain ZMBJ (ZMBJ-CMV)-2b$_{N81}$-STTM vectors successfully downregulaed Nbe-miR165/166 or Nbe-miR159 in *Nicotiana benthamiana*, and Zma-miR167 or Zma-miR482 in maize (Liu et al. 2019).

4.5 Conclusions and Future Perspectives

Viral vectors are widely applied for the analysis of gene function in plants for both forward and reverse genetics. Viral vectors are especially useful when they were inoculated by *Agrobacterium* infiltration, as it is cost effective. Once viral vectors are transformed to *Agrobacterium*, they can infect the plants by means of infiltration. So the cost and time for generating transformants can be reduced. Plants can be infected in the early developmental stage with Agro-drench approach, and the specific tissues, such as fruits, can be inoculated. All the major tissues of the plant can be infected

by viral vectors including leaf, root, flower, and fruit (Table 4.1). The viral vectors have been widely used in studying gene functions.

The non-inheritance was considered as the main drawback of viral vectors. However, recent reports have shown that transient expression of some viral vectors was inherited to the next generation. For instance, there was 10–30% silencing transmission to progeny for *TRV*-VIGS in *Nicotiana benthamiana* and tomato, *CMV*-VIGS in petunia and tomato, and *ALSV*-VIGS in soybean (Senthil-Kumar and Mysore 2011). The silencing transmission was ~50% in pea by *PEBV*-VIGS (Senthil-Kumar and Mysore 2011). The silencing transmission was more than 80% in wheat by *BSMV*-VIGS (Senthil-Kumar and Mysore 2011). Therefore, the stable transgenic plants overexpressing or downregulating target genes can be obtained by taking advantage of the characterization of viral seed transmissibility. Besides, the vegetative tissues, silenced plants can be propagated through tissue culture, callus development, protoplasts multiplication, and other *in vitro* steps (Senthil-Kumar and Mysore 2011).

To date, more than 40 viral vectors have been developed, which are successfully used to silence or overexpress target genes in dicotyledons and monocotyledons (Table 4.1). Some of them have been applied to study miRNA function such as *CaLCuV*, *TRV*, *PVX*, *BSMV*. Other vectors are the potential tools that can be modified for functional analysis of miRNAs. There are 6750 and 2422 miRNAs deposited in miRBase (Release 22.1: October 2018) for eudicotyledons and monocotyledons, respectively. The function of most of them are still unknown. It is promising to uncover the function of miRNAs through viral vectors in a fast and convenient way. Notably, the efficacy of different approaches for inhibition of a given miRNA is different (Wong and Millar 2019). The efficiency of different viral vectors for silencing the target miRNAs are also different (Zhao et al. 2016). Hence, the combination of MIM, STTMs, and SPs with different viral vectors may be taken to maximize the chances of silencing or enhancing the miRNA activity.

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Chapter 5
Use of mRNA-Interactome Capture for Generating Novel Insights into Plant RNA Biology

Naiqi Wang and Anthony A. Millar

Abstract RNA-binding proteins (RBPs) constitute a diverse group of proteins that control the fate and expression of the transcriptome via events collectively termed post-transcriptional gene regulation. They are a relatively understudied class of regulators, where historically the focus has been on gene regulators such as transcription factors and small RNAs. This has been due partly to the inability to globally identify the RNA-binding portion of the proteome. However, this has recently changed with the development of "mRNA-interactome capture"; the UV cross-linking of RNAs to proteins that are in direct contact, followed by the isolation of these protein-RNA complexes and subsequent identification of the RNA-bound proteins by mass spectrometry. In plants, this methodology has now confirmed the RNA-binding nature of 100s of bioinformatically predicted RBPs, as well as the identification of many proteins that were not previously known to bind RNA. Characterizing these RBPs will begin to elucidate the true scope of post-transcriptional gene regulation in plants, revealing novel regulatory mechanisms and biotechnological opportunities for improvement of crop species. We highlight three areas of immediate interest to which this UV cross-linking method can contribute; gene silencing, translational control of protein synthesis during abiotic stress, and the epitranscriptome.

Keywords RNA-binding proteins · mRNA-interactome capture · Gene expression · Abiotic stress · Epitranscriptome

5.1 Introduction: RNA-Binding Proteins Execute Post-Transcriptional Regulation

Gene regulation is fundamental to life, being coordinated via a myriad of molecular interactions that enables the execution of differential gene expression programs that underpin development and responses to environmental cues

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© Springer Nature Switzerland AG 2021

G. Tang et al. (eds.), *RNA-Based Technologies for Functional Genomics in Plants*,
Concepts and Strategies in Plant Sciences, https://doi.org/10.1007/978-3-030-64994-4_5

(Briggs 2016; Hicks 2001). Gene expression commences with the production of RNA via transcription. These RNA molecules are simply carriers of genetic information, needing to interact with cellular factors and machinery in order for them to perform their genetic function. This not only applies to precursor mRNAs (pre-mRNAs) that corresponds to the coding portion of the transcriptome, but also to the non-coding portion, for example, primary-microRNAs (pri-miRNAs). The majority of these cellular factors and machinery correspond to RNA-binding proteins (RBPs), whose complex interaction with the transcriptome determines its fate (Hentze et al. 2018). RBPs not only mediate the processing and modification of RNAs resulting in their maturation, but they also determine expression (translation), localization, and stability (Fig. 5.1) (Obernosterer et al. 2006; Floris et al. 2009; Maldonado-Bonilla 2014; Schwartz 2016). For instance, in the nucleus RBPs mediate the capping of pre-mRNAs at their 5‘ end, and polyadenylation at their 3‘ end. Most RNAs are

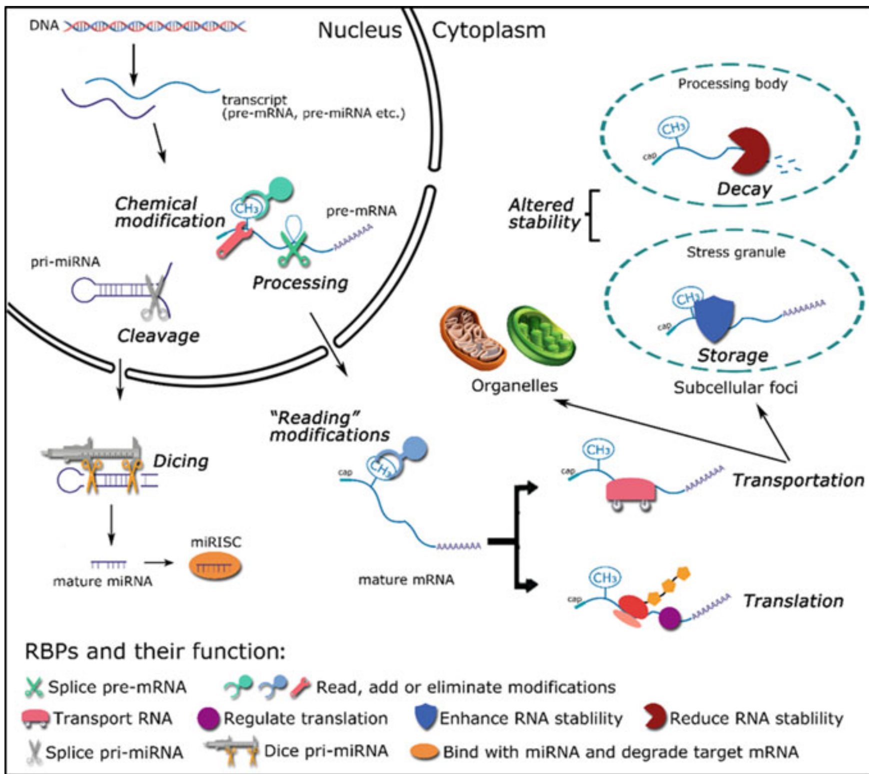

Fig. 5.1 A graphical overview of post-transcriptional regulation. The regulatory processes and effects to transcripts are denoted in bold and italics. The RNA-binding proteins are indicated by cartoons according to their functions

decorated with chemical modifications that are added by RBPs referred to as "epitranscriptomic writers" (Li and Mason 2014; Vandivier and Gregory 2018). Ubiquitously, eukaryotic mRNAs contain introns that are processed by the spliceosome, a complex composed of small nuclear RNAs and proteins including RBPs (Fig. 5.1) (Xiao et al. 2016). Once exported to cytoplasm, mRNAs may be translated or transported to organelles or subcellular foci such as processing bodies (where mRNAs is degraded) or stress granule (where they are protected from translation and decay) (Fig. 5.1) (Chantarachot and Bailey-Serres 2018; Maldonado-Bonilla 2014). "Epitranscriptome readers" and "erasers" also interact with the chemical modifications, controlling RNA fate (Shen et al. 2019). Together these processes are considered post-transcriptional regulation, which ultimately controls the genomic output from a cell, a process that underpins life.

5.2 RBPs Are an Understudied Class of Gene Regulators

Despite this central role in controlling gene expression, RBPs have remained a relatively understudied cohort of gene regulators. One contributing factor to this, is that determining mRNA-protein interaction has remained challenging largely due to limiting technology. Historically, this was in contrast to methods that were available to study other classes of regulators. For instance, RNA-seq makes it relatively easy to identify the global cohort of small RNAs (sRNAs). Supporting these analyses are simple sequence complementary-based programs that predict their targets giving insights into their function (Li et al. 2014). Similarly, methodologies have long existed for the study of transcription factors and their targets. For example Chromatin—immunoprecipitation (ChIP-seq) methodology has been well developed and widely utilized, which again gives functional insight of these regulatory genes.

Consequently, regarding gene regulation, the focus has remained on transcription factors and sRNAs of which many have been functionally characterized. By contrast, for the vast majority of RBPs, little is known about their function, their targets, or even when they are actively binding RNA (Silverman et al. 2013). Confounding this challenge is their heterogeneity. RBPs correspond to a biochemically diverse and complex collection of proteins that interact with RNA via multiple mechanisms, be it RNA sequence motifs, RNA structures, or to the vast array different post-transcriptional chemical epitranscriptome marks decorated on RNA. Defining the cohort of RBPs in a cell, the RNAs to which they bind, and to what structural features they recognize, are all challenging experiments. Consequently, despite eukaryotic genomes contain hundreds of different RBPs (being similar to the number of genes encoding transcription factors), currently our knowledge on the function of vast majority of these RBPs, or the mechanism by which they operate, remain unknown (Wheeler et al. 2018; Lee and Kang 2016).

Of the few RBPs that have been characterized in plants, they have been shown to play crucial roles in development, including flowering (Lim et al. 2004), senescence (Wu et al. 2016), and environmental responses, including circadian rhythms

(Staiger and Green 2011), stresses (Marondedze et al. 2019; Frei dit Frey et al. 2010) and hormones [for reviews see (Bazin et al. 2018; Silverman et al. 2013)]. For example, the FLOWERING CONTROL LOCUS A (FCA) protein harbors an RNA-Recognition Motif (RRM) domain that regulates RNA splicing to suppress target gene expression and promote flowering-time (Lim et al. 2004; Lee et al. 2015). Other RBPs have been shown to play a role in stress response, such as the GLYCINE RICH PROTEINs (GRPs). Their expression is regulated by ABA and they mediate a number of different physiological responses to counter stress (Czolpinska and Rurek 2018). Nevertheless, in plants, much of what is known regarding RBPs is rudimentary and comes via bioinformatic extrapolation from other kingdoms (Silverman et al. 2013).

5.3 The Global Identification of RBPs with mRNA-Interactome Capture

Until recently, our knowledge on which proteins bind RNA came mainly from targeted studies on individual proteins or from bioinformatic predictions of proteins containing known canonical RNA-binding domains (RBDs), as there were no global methods for their determination (Silverman et al. 2013). Attempts to solve this problem included the use of protein micro-arrays (Tsvetanova et al. 2010) or stable isotope labeling by amino acids in cell culture (SILAC) to identify peptides bound to RNA probes (Butter et al. 2009). However, these *in vitro* approaches are limited and may not reflect biologically significant interactions that occur *in vivo*.

Solving this technical limitation has been the landmark development of mRNA-interactome capture, which was pioneered in animal cell lines (Castello et al. 2012; Baltz et al. 2012). Here 254 nm UV light is irradiated onto live cells which covalently cross-links proteins that are directly bound to RNAs *in vivo*, thereby “freezing” mRNA-protein interactions. The advantage of using UV light for cross-linking is that only proteins in direct contact with RNA will form covalent bonds with RNA, and unlike formaldehyde, no protein-protein cross-links will occur, therefore only genuine RBPs are captured (Castello et al. 2012; Baltz et al. 2012). Following cross-linking, mRNA-protein complexes are isolated using oligo(dT) beads. These complexes are stringently washed to remove non-cross-linked proteins. The mRNA-protein complexes are then eluted from the oligo(dT) beads, and then RNA is degraded via RNase treatment, leaving the RNA-bound protein fraction. These proteins are then digested with trypsin and then analysed by quantitative mass spectrometry (MS). Multiple large scale biological replicates are performed on both UV treated [cross-linked (CL)] or non-UV [non cross-linked (nCL)] samples. Proteins that are enriched in the CL sample compared to the nCL sample with strong statistical significance [e.g., a false discovery rate (FDR) of below 1%] are considered strong candidates for being RBPs.

Such an approach captures RBPs in a largely unbiased, systematic manner. Interactome capture experiments have been completed for human HeLa and human embryonic kidney HEK293 cells (Castello et al. 2012; Baltz et al. 2012). mouse embryonic stem cells (Kwon et al. 2013), liver cells, and yeast (Beckmann et al. 2015). Additionally, the approach has been used on whole organisms, such as *Caenorhabditis elegans* (Matia-Gonzalez et al. 2015) and *Drosophila* (Wessels et al. 2016). Together, these experiments have provided experimental evidence of RNA-binding for hundreds of predicted RBPs, which have classical RNA-binding domains (RBDs). In addition, a multitude of other potential RBPs has been identified, that neither have a classical RBD, nor any known association with RNA (Hentze et al. 2018). Therefore, like other unbiased "omics" approaches, the unexpected findings are leading to a paradigm shift in our perception of what an RBP is and what their potential roles in the cell are (Hentze et al. 2018).

5.4 Arabidopsis *in Planta* mRNA-Interactome Capture

The method of mRNA-interactome capture has now been applied to Arabidopsis, including leaf mesophyll protoplasts (Zhang et al. 2016), cell suspension cultures (Marondedze et al. 2016), and an *in planta* study on intact etiolated seedlings (Fig. 5.2; Reichel et al. 2016). These studies have given insights into the portion of the proteome that is RNA-binding. They have provided the first experimental evidence of RNA-binding for 100s of bioinformatically predicted plant RBPs. Additionally, similar to the studies in animals, a large proportion of the captured proteins neither have a classical RBD nor any know association with RNA. This has raised

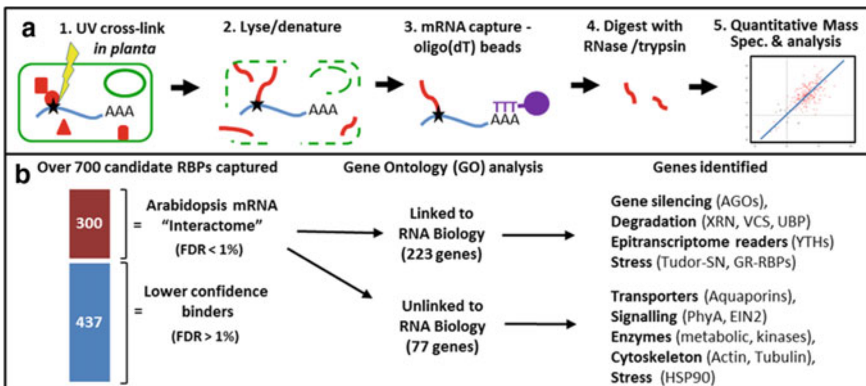

Fig. 5.2 ***In planta* mRNA-interactome capture. a**. Interactome capture [mRNA (blue); UV crosslinks (); proteins (red), oligo-dT beads (purple)]. **b**. Number of identified proteins that are linked or unlinked to RNA biology, including examples

the possibility of identifying many new RNA regulatory pathways and mechanisms that had not been previously considered (Koster et al. 2017; Bach-Pages et al. 2017).

For the Arabidopsis *in planta* seedling study, 737 proteins were captured, of which 300 were enriched in the CL compared to the nCL sample, with a false discovery rate of below 1%. This set of proteins was defined as "interactome RBPs." The remainder of the proteins (437) did not meet these stringent criteria and were classified as "candidate RBPs," which are of lower confidence but still likely to bind to RNA. Gene ontology (GO) analysis revealed that approximately 74% of the interactome RBPs and 46% of candidate RBPs had GO annotations linking their function to RNA, demonstrating that proteins associated with RNA have been preferentially captured (Reichel et al. 2016). Additionally, many of these proteins contained a known RNA-binding domain (RBD); this includes RNA-Recognition Motif (RRM) (80 proteins), K homology domain (12 proteins), DEAD-box helicase domain (12 proteins), pumilio repeats (six proteins), zinc finger types (19 proteins), or pentatricopeptide repeats (12 proteins). Well-known RBPs, such as COLD SHOCK PROTEINs (CSPs), GRPs and TUDOR-SN proteins were isolated, along with many housekeeping RBPs such as POLY(A) BINDING PROTEINs, splicing factors and proteins associated with gene silencing, including AGONAUTE family members (AGO1, AGO2, and AGO4) (Table 5.1). Additionally, a family of ten

Table 5.1 Some examples of proteins identified by the *in planta* mRNA-interactome capture study (Reichel et al. 2016). This includes classes of proteins which have no known RNA-binding function

Gene name	Function	p-value of enrichment
AGONAUTE1 AGONAUTE2	Gene silencing	2.9E-08 1.1E-04
ECT1, ECT2, ECT4, etc.	RNA methylation readers?	7.2E-08 1.2E-13 1.5E-03
Tudor1 Tudor2	Stress	1.5E-15 3.1E-11
GRP5 GRP7	Stress	1.3E-06 6.0E-14
Annexin D4	Stress/secretion	4.9E-07
ACTIN 8	Cytoskeleton	2.4E-04
Tubulin α-4 chain Tubulin β-3 chain	Cytoskeleton	6.6E-06 9.4E-06
Lim protein2B WLim1	Cytoskeleton	9.9E-11 8.4E-11
Aquaporin PIP2-1 Aquaporin PIP2-2 Aquaporin PIP2-7	Intrinsic membrane water transporter proteins	2.0E-05 8.7E-03 5.4E-05
Phytochrome A	Photoreceptor	1.4E-03
Phototropin-1	Photoreceptor	3.4E-03
EIN2	Ethylene signaling	3.5E-09

YTH (YT521B-Homology) domain-containing proteins were captured, also known as EVOLUTIONARY CONSERVED C-TERMINAL DOMAIN family proteins. These proteins are homologous to mammalian proteins that bind the most prevalent mRNA modification, adenosine 6 methylation (m^6A), and are considered part of the epitranscriptome, with the ECT2 protein been shown to increase the stability of its target mRNAs (Wei et al. 2018). Additionally, ECT2 and ECT3 have now been demonstrated to recognize the m^6A mRNA modifications in Arabidopsis, and functional analysis has shown that they control developmental timing and morphogenesis in Arabidopsis (Arribas-Hernandez et al. 2018). As these proteins are redundant with one another, it likely explains why they have not been previously identified with these phenotypes in mutant screens, an issue that is likely common among plant RBPs, as most belong to small to medium protein families (Arribas-Hernandez et al. 2018; Scutenaire et al. 2018).

5.5 The Use of mRNA-Interactome Capture to Address Key Areas of Plant Biology

Gene regulation at the translational level remains enigmatic. Given the ease at which mRNA levels are measured with RNA-seq, gene expression is predominantly quantified via transcriptomics, with the underlying assumption that transcript abundance acts as a proxy for protein levels. However, the plethora of post-transcriptional gene regulatory (PTGR) mechanisms means that the correlation between an mRNA's abundance and its corresponding protein's abundance is poor. In mammalian systems, mRNA levels only account for approximately 40% of the variability in protein levels, with translation efficiency the best predictor of protein expression (Schwanhausser et al. 2011). Moreover, although discrepancies between mRNA and protein levels are designated "translational control," our understanding of the mechanisms behind such regulation is virtually non-existent. For instance, despite the intense focus on plant microRNAs (miRNAs), no unifying theme has as yet emerged of how they mediate repression of their targets via a translational mechanism (Axtell 2017).

Gene silencing. Firstly, ARGONAUTE (AGO) proteins, mediators of gene silencing, have been successfully cross-linked to mRNA (Reichel et al. 2016). For the *in planta* interactome, AGO1 and AGO2 were identified in the "interactome RBPs" (Table 5.1), and AGO4 in the candidate RBPs. In animals, miRNA target genes have been identified in numerous studies through cross-linking and immunoprecipitation of AGO complexes, followed by high-throughput sequencing of RNA (often referred to as HITS-CLIP or CLIP-seq) (Chi et al. 2009; Zisoulis et al. 2010). No such experiments have been achieved yet for plant systems, but this mRNA-interactome result implies this is possible, raising new opportunities to explore which mRNAs are being targeted by the different gene silencing effector proteins (pathways) in plants. Moreover, comparison of an AGO1 CLIP-seq to degradome data will give insights into silencing mechanisms by determining which targets are cleaved (present

in degradome), compared to targets being translationally repressed (targets present in CLIP-seq data, but no degradome signature). Given the ongoing investigation into gene silencing, the mechanism by which it works, and the genes it targets, application of such methodology to plants would be highly significant to the field.

RBPs and selective translation during abiotic stress. Gene expression reprogramming during abiotic stress underpins a plant's response and tolerance. This includes strong gene regulation at the translational level, which occurs during a wide range of stresses, including heat, cold, hypoxia (waterlogging), and water deficit (Merchante et al. 2017). Here, often two opposing translational regulatory events occur; a general decrease in global translation rates, coupled with increased translation efficiency of a select group of mRNAs required for stress survival (Merchante et al. 2017). This occurs as protein synthesis is potentially the most energy-expensive process in the cell; after translation, correct folding, modification, and transportation ensues (Roy and von Arnim 2013). Therefore, regulating what fraction of the transcriptome is translated is a key regulatory step enabling a rapid response to environmental perturbations while conserving energy (Matsuura et al. 2010). In the extreme cases of anaerobic or heat shock, the majority of cellular mRNA polyribosomes dissociate resulting in inhibition of general protein synthesis, while a small group of mRNAs required for stress survival are selectively translated (Minia et al. 2016). For anaerobiosis, enzymes involved in anaerobic metabolism are selectively translated, presumably to make enough ATP to survive the stress (Sachs et al. 1980). Thus, this post-transcriptional gene regulation not only couples a rapid response with energy conservation, but also focuses translation on a subset of proteins to maximize stress survival. Despite this hypoxic response being discovered over 35 years ago, the molecular mechanisms that underlie selective translation during hypoxia, or any other stress, remains unknown. These mechanisms are likely to be complex, but RBPs must be regarded as likely key players (Lorkovic 2009; Ambrosone et al. 2012; Marondedze et al. 2019). Identifying these regulatory RBPs will be central in understanding how these responses occur and may provide opportunities to manipulate them. Indeed, the RBP known as OLIGOURIDYLATE BINDING PROTEIN 1 (UBP1), identified from animal systems via homology, selectively sequesters non-stress-related mRNAs into stress granules during hypoxia to prevent their expression (Sorenson and Bailey-Serres 2014). Other RBPs that are known to play key roles in stress response have already been identified by mRNA-interactome capture during non-stress conditions (Reichel et al. 2016) (Table 1). This includes Tudor-SN proteins that are essential under stress where they stabilizes their targets (Frei dit Frey et al. 2010), and GRPs that are heavily involved in stress response (Czolpinska and Rurek 2018). Elucidating differential RNA-binders between control and stress conditions via mRNA-interactome capture will give the best chance of identifying RBPs that are key in coordinating abiotic stress responses.

The epitranscriptome. Relative to DNA methylation and epigenetics, the epitranscriptome has been poorly studied. This is despite there being over 100 known modifications, inferring there is huge regulatory potential via RNA modification. The most abundant modification is the methylation of adenosine, N6-methyladenosine (m^6A) (Li and Mason 2014), and this modification is added by an RBP referred to as a "writer" (Fig. 5.3). These m^6A modifications are essential for plants, as

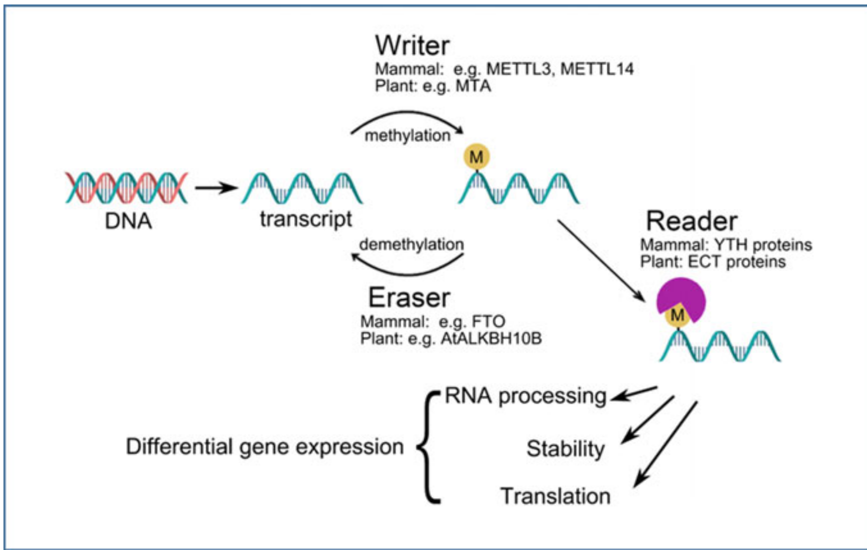

Fig. 5.3 The mechanism of m6A function. Methylation to adenosine on the transcripts is mediated by writers [e.g., METTL3 (methyltransferase like 3) and METTL14 in mammal, MTA (mRNA adenosine methylase) in plant], and can be demethylased by erasers [e.g., FTO (fat mass and obesity-associated gene) in mammal and AtALKBH10B in plant]. Then the m6A is directly interacted by readers (YTH proteins) which lead the transcripts to different processes

mutations in the writer, the RNA m^6A methylase enzyme, are embryo lethal (Zhong et al. 2008). Recognition of m^6A modified RNA is achieved by RBPs referred to as "readers" (Fig. 5.3). Their identity has been determined in animal cells as proteins containing an YTH domain, which binds to m^6A modified mRNA facilitating their degradation (Wang et al. 2014), splicing (Xiao et al. 2016), or translation (Yang et al. 2018). In contrast to humans which only have five YTH domain-containing genes, Arabidopsis has 12 different YTH domain proteins (11 ECT proteins and CPSF30), ten of which were identified in the *in planta* mRNA-interactome (Reichel et al. 2016), confirming that these proteins are binding to mRNA *in vivo*. *ECT2* and *ECT3* have subsequently been demonstrated to regulate the branching of the trichomes, and together with ECT4, are required for leaf developmental timing and morphogenesis (Arribas-Hernandez et al. 2018; Scutenaire et al. 2018). *ECT2* has been confirmed to stabilize the mRNAs related to trichome morphogenesis, and may also regulate the 3'UTR processing (Wei et al. 2018). Additionally, many *ECT* genes are strongly induced by stress, potentially linking the epitranscriptome to stress (Arribas-Hernandez et al. 2018; Scutenaire et al. 2018). However, the function of the majority of the m6A regulators in the plant kingdom is still unclear (Reichel et al. 2019) Therefore, it is likely that we are only beginning to understand the impact of the epitranscriptome, and how it controls genomic output during development and environmental response.

5.6 Conclusions

Plant mRNA-interactomes will open up many new avenues of research that will likely elucidate post-transcriptional gene regulatory mechanisms not previously considered. Full development and exploitation of the methodology will serve as an exhaustive resource for the plant biology community, enabling researchers working on other plant (crop) species to adapt the methodology that has been pioneered in Arabidopsis. We believe interactome capture will be of great interest to the plant scientific community; as has the development of next-generation sequencing revolutionized the field of transcriptomics resulting in an intense focus on sRNA biology, we anticipate that enabling the global, unbiased analysis of the interactome will facilitate such a focus for plant RBPs.

Funding Information This project was supported from funds from the Research School of Biology (RSB), ANU.

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Chapter 6
Slicing Messengers by Artificial Designs: Artificial MicroRNA Induced Gene Silencing in Polyploid Plants for Functional Genomics and Trait Modification

Anandita Singh and Sandip Das

Abstract Evolutionary history of angiosperms illustrates extensive and recurrent whole genome duplication (WGD) events. A direct consequence of WGD is establishment of multiple notional sub-genomes within the polyploid cytotypes accompanied with an overall increase in gene copies known as homeologs. Even in diploids, prevalence of multiple, redundantly functioning gene copies is not unusual and is reminiscent of ancient genome duplication events. Functional analysis of such redundant genes poses challenges while using conventional loss- and gain-of-function approaches. Whereas loss-of-function approaches involving withdrawal of gene function entail recombining homozygous mutant alleles at multiple homeologous loci, serial analysis of gain-of-function mutants generated by over-expressing individual gene copies is cumbersome yet important for delineating homeolog-wise contribution to the phenotype. Development of transgene-based gene silencing technologies provided useful alternatives for functional genomics in polyploids. MicroRNAs and small interfering RNAs (siRNAs) were discovered as key regulators of gene expression based on their ability to base-pair with transcripts in a sequence-specific manner. Such a binding down-regulates target genes via transcript cleavage or translation repression, hence the term RNA interference (RNAi). Since mutants mimic loss-of-function phenotypes, siRNA-based gene silencing tools were initially applied in functional genomics and trait modification in plants. However, such prototypes of RNAi technology suffer from widespread off-target silencing. Artificial miRNA-based silencing platform was designed to enhance specificity and minimize off-target silencing for achieving systematic characterization of genes.

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G. Tang et al. (eds.), *RNA-Based Technologies for Functional Genomics in Plants*, Concepts and Strategies in Plant Sciences, https://doi.org/10.1007/978-3-030-64994-4_6

Through a flexible format, artificial miRNA technology permits specific and efficient silencing of a single or multiple genes by modulating the spectrum of target transcripts. Furthermore, instances of "off-target" silencing are minimized since homogenous population of mature miRNA are more precise relative to heterogenous siRNAs. In refined versions, suitable promoters have been used to regulate expression of artificial miRNAs in select spatio-temporal domains. Constant addition of novel features underpins evolution of artificial miRNA technology and justify its adoption in large-scale gene function studies and trait manipulation. Herein, we provide an overview of the genesis and application of artificial miRNAs to illustrate the impact of the technology over a decade in plant research and crop improvement.

Keywords Artificial microRNA · Gene silencing · Trait modification · Functional analysis

6.1 Introduction

6.1.1 Polyploidy, Gene Redundancy, and Challenge of Functional Characterization

Gene redundancy is all pervasive and is known to have co-evolved with multicellularity with more complex genomes retaining a higher number of gene copies. Especially in plants, evolutionary events such as Whole Genome Duplication have been widespread resulting in polyploidy. Reconstruction of angiosperm phylogeny based on whole genome sequences reveals cycles of genome duplication events followed by gene loss (Mach 2019; Michael 2014). Prevalence of multiple subgenomes in a single plant species is therefore not unusual. While expansion of gene copy number (homeologs) is a direct outcome of polyploidy, plant genomes as such are extremely dynamic (Pennessi 2011) to the extent of being described as "practising anarchy" as quoted by Detlef Weigel (Max Planck Institute for Developmental Biology in Tübingen, Germany). Rampant DNA rearrangements including segmental and chromosomal duplications have further contributed to gene redundancy in both diploid and polyploid plants. Gene duplication sets the stage for sequence and functional diversification. Relieved of functional constraints, duplicated genes often trace divergent evolutionary trajectories to acquire novel or modified functions. Much of the morphological diversity in critical traits apparent in cultivated crops has been established as a consequence of functional diversification of homeologs during the course of crop evolution. In the post-genomic era, a fundamental requirement for crop improvement is knowledge-base on biological role of genomic sequences. Functional genomics in polyploid crops, replete with a vast array of redundant gene copies, is however, not straight forward. Though gene disruption methods such as classical chemical and physical mutagenesis, T-DNA and transposon tagging, constitute a gold-standard for functional genomics, examination of loss-of-function phenotypes is not tenable for redundantly functioning genes. For

this very reason, complete withdrawal of protein function is extremely challenging in polyploids. Furthermore, fixing mutated alleles to homozygosity at each locus followed by recombining these in a common genetic background is a genetic feat not feasible in scenarios wherein redundant genes are tandemly organized. By comparison, gain-of-function mutagenesis involving strong constitutive promoters such as 35SCaMV, is a preferred method for functional characterization of redundant genes. Therefore, in polyploids, individual homeologs are required to be systematically over-expressed to dissect the contribution of each to the phenotype. Pending this, functional characterization of gene homeologs is incomplete. Theoretically, different homeologs may contribute quantitatively to the overall function; few homeologs may evolve novel functions or even be pseudogenised (Jain et al. 2018; Zhang et al. 2019a; Lee et al. 2020). A problem commonly encountered in this approach is manifestation of pseudo-phenotypes. Ectopic misexpression of genes in tissues and developmental stages where the gene is naturally down-regulated can complicate inference of true biological role of gene under investigation.

The methodological repertoire of first generation transgene-based gene silencing technologies such as Post-Transcriptional Gene Silencing (PTGS), Transcriptional Gene Silencing (TGS), and Virus Induced Gene Silencing (VIGS) promise simultaneous silencing of multiple gene homeologs (Waterhouse and Helliwell 2003). Thus, these techniques are relevant for functional characterization of gene homeologs in polyploid crops or for duplicate genes in diploid crops. In addition, transgene-based silencing strategies permit sophisticated modulation of target gene expression since it is possible to achieve quantitative and inducible silencing of even alleles, programmable at both developmental and tissue-specific manner. Mechanistically, an underlying commonality in gene silencing-based knock-down methods is post-transcriptional down-regulation by small non-coding RNAs sharing base complementarity with target transcripts. The sequence-specific recognition of mRNAs by small RNA constitutes a critical step in multifarious strategies for gene silencing. One of the prime concerns of deploying small interfering RNA (siRNA) duplexes for PTGS is unintended silencing of non-target genes, commonly termed as "off-target silencing."

Artificial miRNA (amiRNA) technology was developed as a 2nd generation, gene silencing method to achieve specific and efficient silencing. The flexibility of targeting single or multiple genes simultaneously is a unique aspect of this technology. The intelligent design of amiRNA is rooted in the principle of biogenesis of natural miRNAs, recognition and pairing of amiRNA with target transcripts.

This chapter describes the unique aspects of amiRNA technology, its application in functional genomics and trait modification in polyploid genomes with special focus on crops including Brassicas. Written for the benefit of students and researchers, the chapter intends to inform the readers about basic features of miRNAs, mechanism of miRNA mediated target recognition and silencing; the knowledge of which was creatively applied in design of amiRNAs and engineering method for achieving highly specific silencing of target genes. The chapter also covers various amiRNA technologies and representative biological and computational resources for effective amiRNA designs. Interesting applications wherein potential of amiRNAs have been

exploited are discussed. Finally, strength and limitations of amiRNA technology are illustrated with relevant case examples.

6.1.2 Principles of Natural Small RNAs and Gene Silencing Phenomenon

6.1.2.1 Historical Perspective

One of the most celebrated advancement in biological sciences has been the discovery of regulatory small non-coding RNAs (snc RNAs) and elucidation of their role in directing growth, development, adaptations, and genome reprogramming (Borges and Martienssen 2015). The theoretical framework of gene regulation has come a long way since 1969, when Britten and Davidson implicated a diffusible regulator of transcription in eukaryotic gene regulation (Britten and Davidson 1969). That the RNA moieties have a role beyond serving as a template, scaffold, or an adaptor during protein synthesis, however, remained obscure until the phenomenon of RNA induced gene silencing was discovered around 1980s. Since then, small RNAs have gained center stage and are known to lie at the core of gene regulation. The repressive role of small RNAs is now well established (Baulcombe 2004; Bologna and Voinnet 2014) and deviates only slightly from the original postulation of an "activator RNA" in Britten and Davidson model in which RNA was proposed to regulate a battery of genes. Small RNAs are best described as a sculptor who carves out the shapes and contours of stone into a form using chisel and hammer (Bartel 2018). Using a suite of proteins, small RNAs guide the down-regulation of a spectrum of target genes. The fine modulation leads to establishment of a unique spatio-temporal expression patterns that drive various cellular mechanisms. Small RNAs have thus emerged as key players shaping development, phenotypic plasticity, and adaptations. It is widely established that small RNA pathways originally evolved as a cellular surveillance mechanisms for imparting defence against attacking parasitic viruses and transposons and were subsequently co-opted for control of endogenous genes (Jackson 2005; Axtell 2013).

RNA-induced gene silencing is described in adequate detail in both complex and simple organisms suggesting universality of small regulatory RNAs (Mello and Conte 2004). Remarkably, the molecular processes underlying biogenesis of small RNAs and protein machinery that directs silencing of target transcripts are related across organisms. These processes have been variously christened as PTGS in plants, quelling in *Neurospora crassa*, and RNAi in animals (Waterhouse and Helliwell 2003). Historically, the term RNAi was coined for the very first time in the nematode *Caenorhabditis elegans* (Fire et al. 1998) to describe the chemical nature of interfering entity, the dsRNA (double-stranded RNA). Through a set of elegant experiments that involved feeding the worms with sense, antisense, and dsRNA, Craig

Mello (University of Massachusetts, USA) and Andrew Fire at Stanford University, California, USA, provided a compelling evidence on the nature of silencing principle as "dsRNA". In fact, the Nobel prize in physiology and medicine (2006) was accorded to these scientists for "*discovery that dsRNA triggers potent suppression of gene activity in a homology-dependent manner*" (Advanced Information, The Nobel Assembly of Karolinska Institute 2006). These workers demonstrated that most potent silencing of target gene is achieved by dsRNA and not by sense or antisense RNA alone. Incidentally, *Caenorhabditis elegans* also gained prominence in context to discovery of miRNAs (microRNAs). The *lin-4* allele encoding a small RNA (~21-nt), later classified as a miRNA, was found to bear partial complementarity to its target *LIN-14* within the 3′ UTR region (Lee et al. 1993; Wightman et al. 1993). This case was considered as a biological anomaly until the discovery of another short 21-nt non-coding regulatory RNA termed as *let-7*, also from *C. elegans* (Lee and Ambros 2001; Reinhart et al. 2000).

The lobby of plant scientists, however, assert that RNA-induced gene silencing was first described in plants. Although termed variously as PTGS, TGS, homology-dependent gene silencing, and co-suppression, these are partially overlapping RNAi processes (Lindbo 2012). The critical observations for RNAi like phenomenon were reported in *Petunia hybrida* (Napoli et al. 1990). In an attempt to deepen the pigment coloration in petals of petunia, *CHS* (*CHALCONE SYNTHASE*) was over-expressed as a "sense" transgene in native background. Strikingly, variegated petals bearing all shades of pink, purple, violet and white were observed. Crucially, it was demonstrated that "cloned *CHS* gene" mobilized to endogenous genome background as a "transgene," was capable of over-stimulating the gene activity and silencing the homologous endogenous gene. The terms "homology-dependent gene silencing (HDGS)" and "co-suppression" were thus postulated.

Even prior to this, the hypothesis that an antisense RNA expressed from a transgene construct can effectively suppress gene expression was already tested in various organisms such as bacteria (Light and Molin 1982, 1983; Mizuno et al. 1984), slime mold (Crowley et al. 1985), oocytes of *Xenopus* (Melton 1985; Harland and Weintraub 1985), fruit fly (Rosenber et al. 1985), and mammalian cells (Izant and Weintraub 1984; Kim and Wold 1985; Mol et al. 1988). By the year 2005, around 25 cases of small trans-acting RNA regulators were reported in prokaryotes (Gottesman 2004; Vogel et al. 2014). It was shown that RNA:RNA hybrids resulting from base complementarity between sense transcripts and antisense RNAs could efficiently inhibit translation process. Such initial experiments had laid the foundation of antisense RNA-based mutational analysis of gene function.

In plants, proof-of-concept study for RNAi like phenomenon involved observations made in carrot. Herein, super-transformation of carrot protoplasts with an antisense construct triggered transcriptional inhibition of chloramphenicol resistance gene (Ecker and Davis 1986). Several research articles were published subsequently (reviewed in Jorgensen et al. 2006). Through these studies, a conceptual framework emerged which described that gene silencing occurred either at transcriptional (TGS) or post-transcriptional level (PTGS). In the former case, promoter methylation was shown to result in inactivation of transcription (Matzke et al. 1989; Wassenegger

et al. 1994; Park et al. 1996). In the latter case, degradation of transcripts resulted in gene suppression. PTGS was reported in quick succession in various plant systems such as petunia (Napoli et al. 1990; van der Krol et al. 1990; van Blokland et al. 1994), tomato (Smith et al. 1990), and tobacco (de Carvalho et al. 1992). Today, it is well known that PTGS is achieved either via small RNA-directed cleavage of messenger RNAs or inhibition of translational (Axtell 2013).

6.1.2.2 Understanding RNAi Phenomenon and Small RNAs in Plants: Foundations for AmiRNA Design

Plant genomes encode diverse class of small RNAs which are distinguished on the basis of precursor sequence, mechanism of biogenesis, protein machinery that the small RNA associates with, and mechanisms by which target gene expression is suppressed (Axtell 2013; Singh et al. 2018; Zhang et al. 2019b; Millar 2020; Zhang and Hao et al. 2020). Small RNAs confer specificity and potentiate a protein complex, RNA Induced Silencing Complex (RISC), for silencing of target genes (Bartel 2004). The abundance and variation in categories of small RNA in plants suggest that natural variation in components of RNAi pathways, such as Dicers, RNA-dependent RNA polymerases (RDRs), Argonaute proteins, and allied factors have influenced plant adaptations (Borges and Martienssen 2015; Teng et al. 2020). Despite the differences, classes of small RNA share an underlying commonality as key players in partially overlapping silencing pathways which are intricately regulated. In plants, the biological processes are primarily governed by miRNAs and siRNAs (You et al. 2017). In TGS, small RNAs interfere with target gene transcription by methylating DNA and remodelling chromatin. Alternatively, in PTGS, endogenous and exogenous RNAs can be silenced post-transcriptionally via transcript cleavage or translational inhibition (Jones et al. 1999; Vaucheret 2006; Chen 2012; Rogers and Chen 2013; Ossowski et al. 2008; Millar 2020). The siRNAs have been further sub-categorized as small interfering RNAs that form hairpins (hp-siRNAs), natural antisense siRNAs (nat-siRNAs), heterochromatic siRNAs (hc-siRNAs), and secondary siRNAs. The secondary siRNAs have been sub-divided as ta-siRNAs, pha-siRNAs, and ra-siRNAs (Axtell 2013; Singh et al. 2018).

Small RNA and RNAi in plants has been lucidly described and cataloged (Voinnet and Baulcombe 1997; Kasschau et al. 2002; Rajagopalan et al. 2006; Guo et al. 2016; Rosa et al. 2018; Castel and Martienssen 2013; Guzzardo et al. 2013; Wilson and Doudna 2013; Millar 2020; Guo et al. 2020). Overall, RNAi is a multi-step process which is triggered when a long dsRNA is endogenously expressed or ectopically introduced into the cell. The long dsRNA, termed as precursor, bears structural features such as a stem-loop stabilized by base complementarity. Alternatively, pairing of sense and antisense transcripts also generates the precursor.

Once the dsRNA trigger is generated, RNAi process proceeds in two stages-dicing and slicing. The first stage involves biogenesis of small RNA wherein Dicer (RNAse III) crops the precursor to generate small dsRNA (~20–30 bp) with a 2-nt

overhang at 3′ end (Bartel 2004). Given the function, dicers are likened to a microprocessor (Ipsaro and Joshua-Tor 2015). In the second stage, small RNA-guided recognition of target transcripts for cleavage (slicing) or translational inhibition occurs. One of the strands of the small dsRNA (guide) bears complementarity to the target gene(s) while the other (passenger strand) is degraded. The guide RNA associates with Argonaute proteins (AGO) to form RISC which binds with target transcripts to mediate down-regulation. The guide RNA confers specificity to the RISC for target repression.

A particularly vexing issue for contemporary biologists is to delineate the merging boundaries between origin and mode of action of small RNAs. Across species and even within a cell, a large number of distinct classes of small RNAs are uncovered which are being distinguished based on their cellular functions and interactions with the specific category of Dicer for biogenesis. Differences also exist in the mechanisms and pathways that mediate selection of appropriate strand of dsRNA to be loaded onto RISC. Moreover, AGO proteins constitute a family which recruit allied factors to constitute RISC variants. Open questions persist with regard to mechanisms by which categories of small RNAs are sorted for binding with appropriate AGO counterpart. The constituents of RNAi pathway that control the decision on channeling RISCs to direct silencing at level of transcription, transcript cleavage or translational inhibition also being unraveled (Yu et al. 2017; Siomi and Siomi 2010; Ipsaro and Joshua-Tor 2015). In interest of maintaining focus on amiRNA-based silencing technologies, the scope of this chapter is limited to unique features, biogenesis, and control of miRNAs in plants.

6.1.2.3 The miRNAs: Biogenesis and Mechanism of Action

The miRNA genes in plants are typically 20–24-nt in size (You et al. 2017), originate from multiple loci and predominantly located in the inter-genic regions though it is not unusual to find these embedded within introns, exons, or even transposons (Yu et al. 2017; Yang et al 2012; Bartel and Bartel 2003). Most miRNA genes exist as members of gene family giving rise to identical or slightly different mature miRNAs (Kozomara and Griffiths-Jones 2011; Kozomara et al. 2019). Genomic analysis studies have also revealed that members of miRNA gene family are tandemly organized (Rathore et al. 2016). The promoters driving expression of miRNA genes are similar to ones driving protein coding genes recognized by RNA polymerase II (Pol II) (Coruh et al. 2014; Xie et al 2005; Jain et al. 2018). As is the case with eukaryotic mRNAs, a nascent primary miRNAs (pri-miRNAs) is transcribed, modified by capping at 5′-end and polyadenylation at the 3′-end (Xie et al. 2005; Stepien et al. 2016). Mechanistically, mature miRNAs are processed from pri-miRNAs by RNAse III in two steps by a protein complex consisting of (DICER-LIKE1, DCL1), HYPONASTIC LEAVES1 (HYL1), and SERRATE (SE) to release the miRNA/miRNA* (guide/passenger strand) duplexes (Park et al. 2002; Reinhart et al. 2002; Kurihara and Watanabe 2004; Fukudome and Fukuhara 2017). At an ultra-cellular level, these processes occur in nuclei (Fang and Spector 2007; Li et al.

2016). The pri-miRNA forms an imperfect stem-loop with structural features such as proximal and distal stem and several other unstructured regions (Wang et al. 2019). The Dicer complex introduces two cuts at nearly pre-determined sites to give rise to a much shorter stem-loop (80–250-nt) known as precursor miRNA (pre-miRNA). The pre-miRNA is further cropped by DCL1 to release a 21-nt double-stranded RNA commonly called as miRNA/miRNA* duplex (Yu et al. 2017; Zhu et al. 2013). Finally, HUA1 ENHANCER 1 (HEN1) methylates the 3′-terminal by 2′-O-methylation. Apparently, the modification is essential for stabilizing the small RNA by conferring protection from degradation tags such as 3′-uridylation (Xie et al. 2005; Yu et al. 2005; Li et al. 2005). After stabilization, mature miRNA is loaded on to AGO1 for RISC formation. This step is important since only one strand (guide/miR) of the dsRNA duplex is selected for loading by a mechanism which is non-random. The other strand of the duplex (passenger/miR*) is destroyed (Eamens et al. 2009; Iki et al. 2010). The molecular basis of strand selection has been deciphered and is known to be influenced by several factors. For example, the thermodynamic stability at the 5′-end of the double-stranded small RNA is a crucial determinant for selection. In other words, of the two strands, the strand displaying relatively higher AU content or bearing mismatches at the 5′ end is opted for loading onto RISC (Khvorova et al. 2003). Most plant miRNAs have an over-representation of 5′-terminal uridine to facilitate incorporation into AGO while the 19th position, is usually a Cytosine (Eamens et al. 2009; Manavella et al. 2012). The 5′-U is seldom ever seen in the miR* strands and is instead over-represented for 5′-terminal A. Other structural features such as position of bulges within the miRNA/miRNA* duplex structures affect which AGO member would be associated with miRNA/miRNA* (Ren et al. 2014). Intricate details are available that describe miRNA biogenesis and nuclear export with key players being DCL1, DOUBLE STRANDED RNA BINDING1 (DRB1) also known as or HYPONASTIC LEAVES1 (HYL1), and HASTY (Papp et al. 2003; Park et al. 2005) and various other aspects of RISC loading (Baumberger and Baulcombe 2005; Eamens et al. 2009). The miRNAs are distinct from siRNAs which mostly occur in three sizes (21-, 22-, and 24-nt) in plants. In sharp contrast with miRNAs, which originate from distinct genetic loci, origin of varied classes of siRNAs is diverse. The siRNAs may be triggered by exogenous factors, endogenous natural phenomenon, and even as a secondary reaction of miRNA-mediated target silencing. Whereas miRNAs emerge from imperfectly complementary hairpinned precursors representing intra-molecular interactions, the siRNAs originate from long perfectly complementary dsRNAs via inter-molecular hybridizations by hierarchical and redundant activity of several dicers (DCL4, DCL2, and DCL3) (Axtell 2013; Fusaro et al. 2006). Whereas miRNAs act in *trans* and repress activity of genes that are present on distinct loci, siRNAs may suppress gene activity in both *cis* and *trans* (Axtell 2013). Another feature that distinguishes miRNAs from siRNA is the ability of latter to amplify silencing signal by generating “transitive” siRNAs. Sequence analysis of small RNA fractions shows that the secondary siRNAs map to regions within and around the target locus (Himber et al. 2003; Schwab and Voinnet 2010). As mentioned earlier, the two well-defined mechanisms by which plant miRNAs are known to silence target genes post-transcriptionally are transcript

cleavage and/or translation repression (reviewed in Voinnet 2009; Yu et al. 2017). Both these mechanisms rely on miRNA pairing up with target transcript on a specific binding motif termed as miRNA binding site (Jones-Rhoades et al. 2006). Broadly, plant miRNAs demonstrate substantial base-pairing with targets relative to animal miRNAs wherein partial base-pairing also results in down-regulation of target transcripts. The speciality of extensive base-pairing of plant miRNAs has been leveraged for computational prediction of target sequences (Jones-Rhoades and Bartel 2004). The cleavage occurs precisely at a site complementary to position 10/11 on the miRNA with an over-represented A at position 10 (Song et al. 2004). Translational inhibition is the predominant mechanism in animals wherein the miRNA anneals with 3′-UTR regions harboring the binding site despite several mismatches. By contrast, in plants, the miRNA binding site is embedded within the coding sequence and binding requires higher number of matches. Further, experimental studies suggesst that transcript cleavage is the more prevalent mechanism by which miRNAs repress the target genes (Chen 2009; Aukerman and Sakai 2003; Llave et al. 2002; Kasschau et al. 2003; Palatnik et al. 2003). Few miRNAs such as miR172 and miR156, repress respective target genes by both transcriptional cleavage and translational inhibition (Aukerman and Sakai 2003; Chen 2004; Gandikota et al. 2007; Lauter et al. 2005; Mlotshwa et al. 2006; Schwab et al. 2005; Wu and Poethig 2006). Once the target transcript is recognized, it is sliced at a specific position (Llave et al. 2002). The molecular mechanisms of miRNA mediated translational inhibition are still being unraveled. In the following account, the prevailing themes for molecular basis of target selection by miRNA will be discussed which constitutes the basis for design of amiRNAs.

6.1.2.4 Patterns of Natural miRNA Pairing for Selection of Targets: Rules of Target Recognition by amiRNAs

The prime motivation of accurate prediction of miRNA targets has been to enhance development of technologies for intended silencing of genes. Since plant miRNA sequences bear a near-perfect complementarity, computational and statistical analyses combined with comparative genomics permit efficient prediction of miRNA targets (Llave et al. 2002; Reinhart et al. 2002; Sunkar and Zhu 2004). Many of the initial studies showed that for plant miRNAs to effectively recognize target transcripts, the number of mismatches with respective binding motifs must not exceed three (Aukerman and Sakai 2003; Chen 2004, Vaucheret et al. 2004; Jones-Rhoades and Bartel 2004). Experimental analyses of genome-wide expression data of an over-expressing miRNA mutant, however, showed that plant miRNAs could direct cleavage of transcripts despite up to five mismatches (Palatnik et al. 2003). Other sophisticated and systematic experiments based on genome-wide expression profiling were subsequently designed to empirically determine rules for selection of targets by plant miRNAs. The experimental innovation involved tracking one-on-one, interactions of miRNAs with the respective targets using plant transcriptome to

understand and develop parameters that specify recognition of targets by miRNAs (Schwab et al. 2005).

Trend analyses of mismatches using a large number of functional miRNAs and respective targets broadly indicated that complementarity to the 5′-terminal and central region is crucially important though mismatches may be tolerated at the 3′-end (Mallory et al. 2004). The base-pairing at 5′-end, plausibly, enables RNA–RNA hybrid formation which is crucial for efficiency of RISC-mediated cleavage (Ameres et al. 2007). The positions 2-12 of the miRNA, also termed as seed region, are particularly sensitive to mismatches (Schwab et al. 2005). Most natural miRNAs show a perfect binding in this region with a maximum of one mismatch to the target. The single allowable mismatch, however, is never found in the position 10/11 that coincides with the cleavage site. Thermodynamic analysis of binding energy of miRNAs paired with targets suggests low free energy not exceeding 72% of a perfect match. Only one mismatch is tolerated in the region complementary to nucleotides 2-12 of the miRNA, but not at the cleavage site. This implies that a single mismatch at 5′-end of the miRNA does not impair the cleavage so long as the mismatch position is not at 10/11. However, detailed inspection reveals that a combination of several factors may abolish a binding. For example, even though mismatches outside the seed region, at the 3′ end of the miRNA and a single mismatch at 5′ end, alone, do not interfere with binding, but presence of more than two consecutive mismatches at the 3′ end of the miRNA abolished the binding if this configuration was combined with a single mismatch in the 5′ region even if the mismatch in the latter was not at position 10/11 of the miRNA. Similarly, stability conferred by upto 10 matches in the 5′ end, can be off-set by a contiguous run of over three mismatches at the 3′ terminal of the miRNA (Schwab et al. 2005).

6.1.2.5 Conventional RNAi-Based Technologies in Plants

Historically, RNAi-based phenomenon were already recognized for their applicability in functional analyses of unknown plant genes and trait modification (Napoli et al.1990; Jorgensen et al. 2006). One of conventional RNAi technologies derived from PTGS, popularly used in plants till date, relies on triggering production of siRNAs capable of silencing endogenous gene (Watson et al. 2005). The basic trick involves engineering target gene sequence as an inverted repeat in a silencing construct to potentiate the transgene specific long dsRNA into forming a fold-back structure. The potency of such an RNAi construct increased manifold when a natural intron was engineered in splicing configuration to stabilize the hairpin loop (Wesley et al. 2001). Hence the term ihpRNA (Intron spliced hairpin RNAs). Thereafter, several proof-of-principle studies testing the potential of variants of ihpRNA constructs were published (Chuang and Meyerowitz 2000; Wesley et al. 2001; Kerschen et al. 2004; Smith et al. 2000). At CSIRO (http://www.pi.csiro.au/rnai/), vectors (pHANNIBAL, pHELLSGATE, etc.) are commercially available that facilitate easy and high-throughput cloning in silencing constructs for functional genomics.

TGS based RNAi tools are not popular in plants. This method involves generating siRNAs from unique hairpins designed to target non-coding regions in the vicinity of promoters. The overall purpose is to elicit widespread promoter methylation and chromatin modification via RNA-directed DNA methylation (RdDM) to achieve target gene silencing (Matzke and Mosher 2014). RNAi has been employed extensively for crop improvement (Andrade and Hunter 2016; Guo et al. 2016; Tang et al. 2007; Regina et al. 2006). VIGS approaches have also been applied for provoking the production of siRNAs against target genes. In nature, an important aspect of plant protection is ability to target hair-pinned double-stranded viral RNA after infection when the viruses replicate. These dsRNA moieties attract Dicers to produce siRNAs (Molnár et al. 2005). An understanding of these natural mechanisms has made it possible to target the native copy within the plant genome upon viral infection (Voinnet 2005). Overall, the RNAi-based silencing technology is a powerful platform for large-scale functional genomics. The mutant alleles thus generated are dominant in nature and permit easy functional analysis.

6.1.2.6 Challenges of Conventional RNAi Technologies: Lack of Specificity and Unintended "Off-Target Silencing"

The traditional siRNA-based RNAi technologies, although effective, suffer from a major drawback. The siRNAs specific to a target transcript, tend to pair with "unintended targets," albeit with partial complementarity, to trigger potent silencing (Sethil Kumar and Mysore 2011). This results in undesirable non-specific silencing and the phenomenon is termed as "off-target silencing." The first instance of off-target silencing was reported in mammalian cell cultures (Jackson et al. 2003; Aimee et al. 2006). Several studies reported that siRNAs bearing differing degrees of complementarity could silence unintended targets (Lin et al. 2005). Detailed analyses revealed that off-target transcript silencing occurred when seed region of miRNA showed complementarity with the 3′-UTRs of transcripts (Jackson et al. 2006). To this effect, computational tools have been developed for prediction of off-targets during PTGS (Xu et al. 2006). It is speculated that double-stranded self-complementary transcripts may give rise to a large diversity of siRNAs from hitherto undetermined cleavage sites by DCLs. Purely by chance, some siRNAs may have sufficient complementarity to an appropriate sequence context on other transcripts not meant to be silenced, such that all sequence-based determinants and energetic requirements are met. Specifically, VIGS and PTGS-based silencing technologies have often reported off-target silencing which severely limits their applicability in crop improvement (Auer and Frederick 2009). For example, commercial RNAi knock out mutant lines available at stock centers such as AGRIKOLA (http://www.agrikola.org) are likely to suffer from widespread off-target silencing and need to be analysed with caution (Sethil Kumar and Mysore 2011). From the perspective of trait modification and functional genomics, the possibility of off-target silencing needs to be carefully assessed since silencing of unintended transcripts may result in manifestation of unrelated phenotypes. Till date, off-target silencing remains a major challenge while deploying RNAi

technology for crop improvement and functional genomics. Phenotypes resulting from silencing of unknown genes, beyond the intended target, make functional analyses confounding. For example, a mutant displaying multiple phenotypic modifications could suggest both off-target silencing and pleiotropy. Tighter constraints are to be imposed if RNAi-based technology is designed to engineer traits. Instances of off-target silencing can result in inadvertent silencing of essential traits in the genetic background of a crop variety. Until the molecular intricacies underlying siRNA mediated silencing are deeply understood, the RNAi-based technologies must be applied in a conservative manner.

6.1.2.7 amiRNA Technology: The Promise of Specificity and Efficiency

Inspired by the unique mechanisms by which natural miRNAs recognize target transcripts, several scientists posited the possibility of using natural miRNA precursors as a surrogate to achieve processing of an synthetic miRNA sequence to silence any gene of choice. The potential of such a miRNA-based gene silencing technology was envisaged to be far-reaching and impactful relative to other RNAi technologies. Unlike siRNAs, mature miRNAs bind to target transcripts at a specific and defined site on the target transcripts. The mechanistic aspects of sequential release of mature miRNAs by DCL1 from pri- and precursor miRNAs are well understood. Furthermore, the empirical parameters by which miRNA guides RISC to repress target transcripts are fairly well characterized. By comparison, sequence diversity among siRNAs is immense when designed to target a specific transcript. The, heterogenous siRNAs can potentially pair up at different regions within the target transcript. As a result, the specificity of any siRNAs-based gene silencing method is often compromised. Besides, siRNAs are derived from numerous sources, with pathways and molecular determinants presenting a mind-boggling complexity to adapt as a silencing methodology. By contrast, mature miRNAs recognize and bind with only ~21-nt target transcript region rendering miRNAs as more specific. These features suggested the possibility of designing amiRNAs capable of specific and potent silencing of target genes.

The design of amiRNA technology was conceived and successfully implemented by Zeng et al. (2002). These workers validated the theoretical possibility of *in-vivo* production of synthetic miRNAs for silencing a set of target genes in HeLa cell lines. Soon after, amiRNA technology was also demonstrated in model plant species *A. thaliana* (Parizotto et al. 2004) for silencing of reporter gene expression. More examples followed confirming efficiency of up to 90% and specificity of amiRNA technology (Alvarez et al. 2006; Schwab et al. 2006; Choi et al. 2007; Mathieu et al. 2007; Niu et al. 2006; Qu et al. 2007). Today, amiRNA-based silencing protocols are well established and are opted routinely for functional studies and crop improvement.

The principle underlying amiRNA technology is based on the knowledge that proper configuration and secondary structure of the miRNA precursor sequences, including position of mismatch bulges and loops, constitute critical determinants for biogenesis of miRNA–miRNA* duplex. In other words, the DCL1 recognizes

specific and unique structural features on precursor stem loop and not any sequence. Conceptually, therefore, engineering two substitution events that replace the true miRNA and miRNA* borne on natural precursors with synthetic miRNA/miRNA* sequences, while also preserving the secondary structure of precursor, should result in efficient processing of amiRNA/–amiRNA* duplex by DCL1. Thus, amiRNA technology aims at dodging the DCL1 microprocessor to release the amiRNA sequences by embedding these into the scaffold of precursor structures. Designing miRNAs, however, is beyond a mere genetic engineering feat. It uses site-directed-mutagenesis for replacement of natural miRNA and miRNA* sequences on the precursor with artificial ones. Selection of an appropriate candidate sequence that will be processed as a mature 21-nt amiRNA requires an in-depth understanding of the rules that govern specific recognition of targets by natural miRNAs while preventing any inadvertent silencing of unintended targets. A favorable feature of amiRNAs is that their expression can be regulated by suitable promoters to achieve down-regulation of targets in pre-defined spatio-temporal domains. The amiRNA technology gained popularity as it avoids problems encountered in siRNA-based technologies. The PTGS-based methodologies often failed either on account of inadequate expression of siRNAs or inability of siRNAs to bind with targets. Further, feedback regulation of target genes for maintenance of steady state levels of target transcripts also presented undesirable outcomes. The sophisticated design of amiRNAs circumvents most of such difficulties. The sequence of amiRNAs can be carefully chosen and optimized for properties that favor efficient silencing of small RNAs. The specificity of amiRNAs and its programmable format to achieve both specific and/or redundant silencing of target transcripts has provided immense flexibility. A key advantage of amiRNA technology has been accuracy and efficiency of silencing. In plants, a single gene is targeted by a single miRNA. In contrast, in animals, multiple miRNAs may target a single gene (Axtell et al. 2011; Krek et al. 2005). This has been the guiding principle for designing and employing a amiRNA for silencing of a "single" plant target gene, an approach termed as "single-hit" amiRNA (Schwab et al. 2006; Ossowski et al. 2008; Teotia et al. 2016). The various design tools generally predict multiple amiRNA sequences that need to be tested for performance. Experimental findings reveal that silencing efficiencies of these amiRNAs, if compared, may vary (Hu et al. 2014; Sharma et al. 2019). As a solution, a "two-hit" amiRNA with multiple amiRNAs for the same target gene has been recommended to achieve maximal silencing efficiency (Teotia et al. 2016).

Few leading research groups have generated automated web-based free-ware (WMD, Web-based MicroRNA Designer, http://wmd2.weigelworld.org; P-SAMS; Table 6.1) to facilitate even beginners to design miRNAs against the gene or genes of interest using a set of parameters (Schwab et al. 2006; Carbonnel 2017, 2019). The only requirement for designing an efficient and specific amiRNA, however, is availability of whole genome transcriptome data to screen out candidates with potential to trigger off-target silencing. The robustness of such tools increases with availability of transcriptome data. The tools may even benefit from other databases such as EST collections available in public domain (e.g. Guo et al. 2020; Kozomara et al. 2019). While designing work-flow (discussed in following sections in detail), care is taken to

Table 6.1 Artificial miRNA tools and resources

S. No	amiRNA designer resource site/tool	Remarks	Reference/website
1	WMD3 http://wmd3.weigelworld.org/cgi-bin/webapp.cgi	One of the first amiRNA designer tool; permits amiRNA prediction against a large variety of organisms and has a wider use	Schwab et al. (2006)
2	P-SAMS http://p-sams.carringtonlab.org/	Web-based amiRNA and synthetic tasi-RNA designer tool for gene silencing in a wide variety of plants	Fahlgreen et al. (2016)
3	http://smallrna.mtu.edu/dna_vector/submit.htm (MicroRNA designer)	Allows selection of miR157 or miR168 to be used as backbone for amiRNA synthesis. Design is limited by sequence only and does not allow use of gene IDs. The site does not have any genome/expression dataset to compare the query sequence	http://smallrna.mtu.edu/dna_vector/submit.htm
4	amiRNA designer http://www.cs.put.poznan.pl/arybarczyk/AmiRNA/	Semi-automated, downloadable off-line program	Mickiewicz et al. (2016)
5	miRNA-SONG https://www2.med.muni.cz/histology/miRNAsong/	A web based tool for designing of “sponges” for sequestration of miRNA	Barta et al. (2016)
6	miR-Synth http://microrna.osumc.edu/mir-synth	Designed specifically for humans, mouse and rat. Need to upload target and non-target sequence/s (up to 8)	Laganà et al. (2014)
7	https://labs.biology.ucsd.edu/schroeder/phantomdb.html	amiRNA library collection for *Arabidopsis thaliana*	Hauser et al. (2013, 2019)

implement principles and features of natural miRNAs to achieve maximum efficiency and specificity. The remaining section will provide details using WMD platform as a case example. The WMD tool facilitates generation of amiRNAs-based silencing constructs in two steps. In the first step, amiRNA candidates are selected and ranked according to predicted efficiency and specificity. This entails *in silico* scanning of target transcript(s) for selection of an optimal region against which, if an amiRNA is designed, the interaction would meet maximum number of criteria that govern interaction of a natural miRNA with its target. The software selects 21-nt potential regions after scanning the reverse complement of entire transcript while ensuring several parameters. Most importantly, the position 10 of the amiR must be an A. The

predicted amiRNA must display 5′ instability implying AU content to be higher relative to 3′ end which must ideally be GC rich especially around position 19. The WMD tool intentionally adds a U at 1st position of 5′ end even in cases where base-pairing rules predicts other nucleotides. The selected candidates qualifying these coarse criteria are subjected to fine screening. Iterative *in silico* mutations, specifically at two regions, viz., 13-15 and 17-21, are created to simulate the hybridization characteristics to a defined target transcript while ensuring that more than two mismatches do not occur as consecutive runs. The software is programmed to select amiRNAs that show perfect match in the seed region (position 2-12 of the amiRNA). However, a single mismatch may be tolerated if sequence context of targets does not present an ideal region. The stringency at position 10 and 11, coinciding with slicing site is much higher and any mismatch on this position is disallowed. A maximum of four mismatches are allowed in the 3′ region around position 13-21, however, over two adjacently positioned mismatches in this region are rejected. The software also imposes empirically determined parameters for thermodynamics. The free energy ΔG of binding of predicted amiRNA should be at least 70% of a perfectly matched duplex and at least 30 kcal/mol. These concepts have been derived from RNAcofold (Bernhart et al. 2006) and mfold (Zuker 2003). If an amiRNA is to be designed to silence multiple transcripts, the constraints will be greater with trade-offs. In such cases, the interactions with the subsequent transcripts may be less stringent. Additional finer aspects include intentional introduction of mismatches between positions 17-21. This is done to avert transitivity.

In the second step, the WMD tool aids a researcher in designing primers via an oligo-designer tool to facilitate exchange of the natural miRNA–miRNA* with the artificial counterparts predicted in the step 1. To enable engineering of selected candidate for amiRNA and amiRNA*, the precursor for miR319a cloned in a routine plasmid vector is to be used. Other precursor backbones may also be used as demonstrated in several studies involving *MIR164b*, *MIR159a*, *MIR171*, and *MIR172a* (Alvarez et al. 2006; Niu et al. 2006; Parizotto et al. 2004; Schwab et al. 2006, respectively). Oligo-designer predicts four sets of primers, for carrying out overlapping PCR on the precursor backbone which finally results in replacement of miRNA–miRNA* with amiRNA: amiRNA*. Three sets of primers are used in distinct PCR reactions using the vector backbone lodging the precursor as a template. The regions to be amplified 5′ sequences covering amiRNA*, the loop including sequence corresponding to amiRNA* up till amiRNA, and 3′ region including amiRNA. The three amplicons bear overlapping sequences (25 bp) corresponding to amiRNA and amiRNA* and can therefore be easily fused in the 4th PCR reaction to reconstitute a precursor harboring amiRNA: amiRNA*. The silencing construct thus generated may be shuttled into a suitable plant transformation vector and introgressed into the plants.

Once transgenics have been generated, the efficacy of the amiRNAs needs to be validated. This involves detection of mature miRNA which is indicative of proper biogenesis from the mutated precursor backbone. For this, small RNA-based Northern blots using amiRNA specific probes may be used (Aukerman and Sakai 2003). Alternatively, stem-loop primed reverse transcription method proposed by

Chen et al. (2005) may be adapted for detection of amiRNAs. Transcript cleavage may also be detected by RACE-PCR (Llave et al. 2002). Quantitation of decreased transcript levels may also be tested by designing RT-PCR primers spanning the presumptive cleavage site. Further, the stem-loop PCR-based method devised for detection of miRNAs may be used for detecting cleaved transcripts (Tyagi et al. 2018, 2019; Dhakate et al. 2014; Shivaraj and Singh 2015). It may be worthwhile to first test the efficiency of amiRNAs using transient Agro-co-infiltration assays in *N. benthamiana* as described by De Felippes and Weigel (2010), since generation of stable transgenics is challenging.

In the following section, work-flow of popular web-based platforms will be discussed.

6.2 Artificial microRNA Based Tools: Design and Engineering

Several tools have been developed and are available as public resources for designing and engineering of amiRNA against candidate gene/s in plants and salient features of a select few are discussed below. Table 6.1 provides a representative list of web and software resources that have been developed for designing amiRNA for gene silencing. A variety of microRNA precursor sequences have been employed as backbones for engineering of amiRNA, the primary criterion being high levels of endogenous expression levels. Since the mechanism of biogenesis of miRNA is highly conserved across organisms, heterologous systems for engineering of amiRNAs have been used. However, usage of precursor backbones from native system for engineering of amiRNA is preferred (Cantó-Pastor et al. 2015), *Gossypium* (Ali et al. 2013), *Medicago truncatula* (Devers et al. 2013), *Malus domestica* (Charrier et al. 2019), *Marchantia polymorpha* (Flores-Sandoval et al. 2016) (Table 6.2).

6.2.1 Web MicroRNA Designer

One of the first tools that was developed is the WMD tool. The current version (WMD3) is versatile and user friendly (http://wmd3.weigelworld.org; Schwab et al. 2006). The web-resource is free-to-use and allows researchers to design and engineer miRNAs against a wide variety of plants over a wide taxonomic spectrum. The following section provides a step-wise guide for using the WMD3 tool. Five tabs, viz., Target search, Designer, Oligo, Hybridize, and BLAST, constitute the core tools for amiRNA design which are described below (Fig. 6.1). Besides, important information may be accessed from Help and Download section.

Table 6.2 Precursor sequences used as backbone for engineering of artificial miRNAs

S. No.	*MIRNA* precursor	Plant source	Vector(s)	Reference
1	*MIR319*	*Arabidopsis thaliana*	pRS300	Schwab et al. (2006)
2	*MIR528*	*Oryza sativa*	pNW55	Warthmann et al. (2008), Yan et al. (2012)
3	a. *MIR390* b. *OsMIR390* + *AthMIR390a*	a. *O. sativa* b. Chimeric precursor proximal sequence from *O. sativa* and distal stemloop end from *A.thaliana*		Carbonell et al. (2015)
4	*MIR390a*	*A. thaliana*	AtMIR390a-B/c vectors	Carbonell et al. (2014)
5	*MIR1157*	*Chlamydomonas reinhardtii*	pChlamiRNA1 and pChlamiRNA2	Molnar et al. (2009)
6	*MIR168a*	*A. thaliana*	pPZamiR-2 POT series	Bhagwat et al. (2013)
7	*MIR395* polycistronic precursor	*O. sativa*	–	
8	*MIR159b*	*A.thaliana*	–	Belide et al. (2012)
9	*MIR159a*	*A.thaliana*	–	Lafforgue et al. (2013)
10	a. *MIR319a* b. *MIR528*	a. *A. thaliana* b. *O.sativa*	Universal vector pUA	Zhou et al. (2013)
11	*MIR156d*	*Malus domestica*	–	Charrier et al. (2019)
12	*MIR156, MIR164, MIR171*	*A.thaliana*	pEARLY GATE	Liang et al. (2019)
13	*MIR164*	*Triticum aestivum*	–	Gasparis et al. (2017)
14	*MIR528*	*O. sativa*	pTAC	Li et al. (2014c)
15	*MIR319a, MIR395a*	*A.thaliana*	pMIR319a;pMIR395a	Liang et al. (2012)
16	*MIR319e*	*Vitis vinifera*	–	Castro et al. (2016)
17	*a. MIR160* *b. MIR166*	*a. Marchantia polymorpha* *b. Selaginella kraussiana*	–	Flores-Sandoval et al. (2016)

(continued)

Table 6.2 (continued)

S. No.	*MIRNA* precursor	Plant source	Vector(s)	Reference
18	*MIR169a*	*Gossypium*	–	Ali et al. (2013)
19	*MIR159b*	*Medicago truncatula*	–	Devers et al. (2013)
20	*MIR408*	*Populus trichocarpa*		Shi et al. (2010)
21	*MIR166*	*Lemna gibba*		Cantó-Pastor et al. (2015)
22	*MIR319a*	*Nicotiana benthamiana*	*MIR VIGS* (artificial miRNAs in VIGS)	Tang et al. (2010)

Target search: The basic purpose of this tool is to align any short sequence with the transcriptome or EST databases of the multitude of databases representing nearly the entire spectrum of plant kingdom. The tool is based on GenomeMapper developed as a part of 1001 genome project (http://www.1001genomes.org). This feature has been recruited to scan the transcripts for potential match with candidate miRNAs, endogenous or artificially designed. The target search parameters allow users to optimize the selection of candidates by simulating various parameters such as number of mismatches, dG (kcal/mol), hybridization temperature, strand selection, and indels. The output is a list of gene identifiers corresponding to the transcripts ranked based on dG values.

Designer: The designer tab allows users to design specific amiRNAs against one or multiple transcripts present in the transcriptome databases. The user may provide information about candidate gene(s) in various input formats such as gene ID or as FASTA sequence format. Gene ID may be retrieved using the BLAST tool implemented within WMD3 to identify homologs. User may also provide FASTA file of transcripts that are not part of any transcript database such as marker genes. WMD3 also permits users to design and silence splice forms of transcripts using amiRNAs. This entails providing the splice-form specific sequence as FASTA file in the input window and adding the gene IDs of non-target splice-forms as off-targets. For designing of amiRNA against multiple genes, the number of genes to be silenced has to be entered in "acceptable off-target" window. BLAST or Target search tools could be used to retrieve gene IDs, and multiple genes with similar sequences.

The designer window also provides options for choosing the "minimum number of targets to be included," and "accepted off-targets" for amiRNA-mediated silencing. To ensure gene-specific silencing, the "off-targets" are recommended to be kept at "0." In case members of gene family or paralogous copies from polyploid genome are to be redundantly silenced, the number of homologs as candidate targets needs to be identified based on BLAST search. The output of Designer tool is received as an email-link and contains a list of amiRNAs ranked based on multiple criteria including degree of 5′ instability, hybridization energies to perfectly matched target (no mismatch) and intended target (may contain mismatch), number and position(s) of mistmatch, pairing with other non-target gene(s) with up to five mismatches.

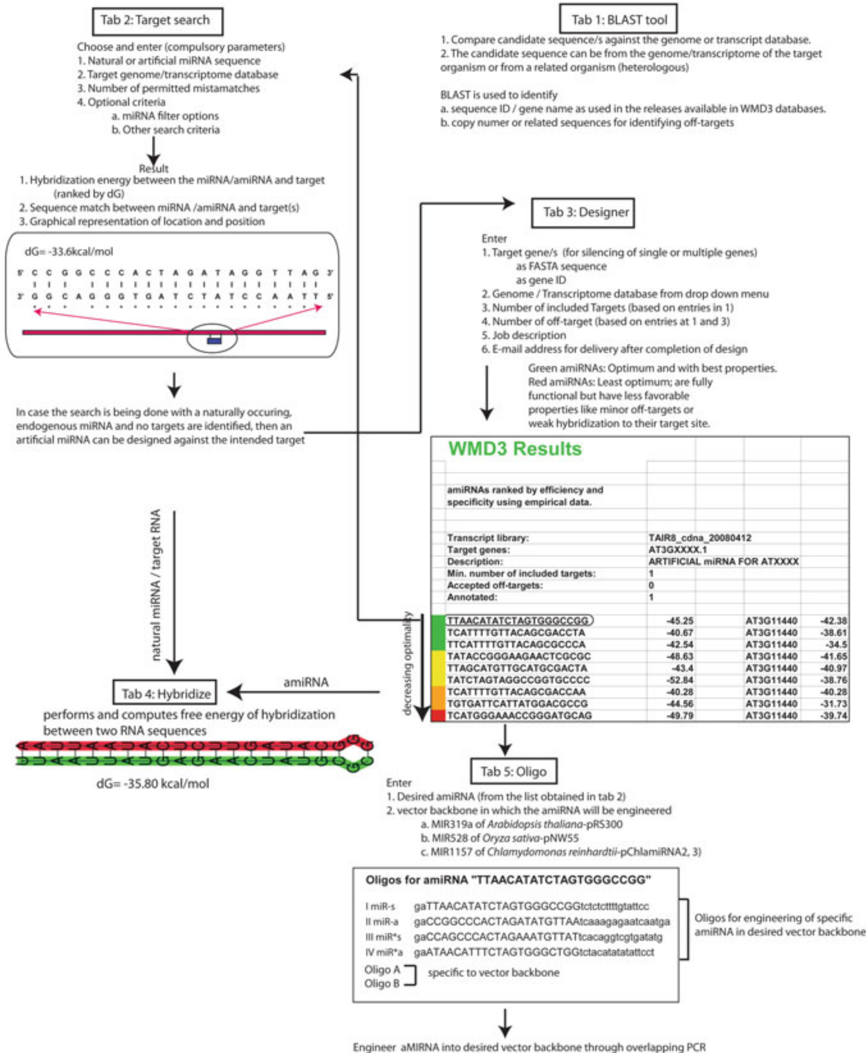

Fig. 6.1 Schematic representation of workflow of Web MicroRNA Designer (WMD3) tool for design of artificial miRNAs. The WMD3 program integrates several tools viz. Target Search, Designer, BLAST, Hybridize, and Oligo. The results obtained from BLAST and Target search may be used to optimize the amiRNA design parameters including number of included and off-targets in the Designer tool. The output obtained from Designer tool can be used to identify targets using Target Search, evaluate hybridization kinetics using Hybridize tool, and design primers for amiRNA engineering using Oligo tool. The workflow between the various tools are interlinked, and marked with solid directional arrows (black)

The selection of amiRNA from the list should be further based on criteria such as "no mismatch between position 2-12 of the amiRNA," "1-2 mismatches between position 18-21 of the amiRNA," "hybridization energy to range between −35 to −38 kcal/mol," "target-site position," and "mismatch pattern for all intended targets for redundant silencing of multiple targets."

Oligo: Once the user has selected an appropriate amiRNA, it needs to be engineered into an appropriate *MIRNA* precursor using overlapping PCR. This is accomplished via four PCR reactions using two precursor-specific oligos, and four oligos that span the amiRNA and precursor. The overlapping PCR results in substitution of native mature miRNA and miRNA* sequence with amiRNA and amiRNA*. WMD3 tool allows users to select precursor backbone of *MIR319a* of *Arabidopsis thaliana* (pRS300 vector; Schwab et al. 2006), *MIR528* of *Oryza sativa* (pNW55 vector; Warthmann et al. 2008) or *MIR1157* of *Chlamydomonas reinhardtii* (pChlamiRNA2, 3 vector; Molnar et al. 2009). The oligos spanning the amiRNA with a specific precursor backbone can be designed by first selecting the desired precursor backbone and then entering the selected amiRNA in the input window which returns back the four oligos for use as primers. The oligos contain the amiRNA, and their complementary sequence flanked by sequences complementary to the precursor to facilitate overlapping PCR for engineering (Table 6.2; Fig. 6.1).

6.2.2 The Plant Small RNA Maker Suite (P-SAMS)

The P-SAMS was developed by Carrington group and tool has two options, to design an amiRNA or to design synthetic tasi-RNA (syn-tasiRNA) (http://p-sams.carringtonlab.org/; Fahlgren et al. 2016). The amiRNA designer allows design of amiRNA against candidates available in transcript database(s) of 24 plant species including algae, dicots, and monocots (Table 6.3). The designer is optimized to predict primers for engineering of amiRNA in *MIR390a* precursor of *Arabidopsis thaliana* or *MIR390* precursor of *Oryza sativa.* Alternatively, if the user already has amiRNA sequences, then P-SAMS tool allows primer prediction for cloning purposes (Fig. 6.2a).

The work-flow for designing of amiRNA begins by the user selecting a transcriptome database. Alternatively, the user may instead use a FASTA file of candidate transcript for prediction of amiRNA. In the former scenario, further options of use of either a gene ID or FASTA sequence file of candidate transcript is available. Either way, by entering multiple IDs or multi-FASTA file, it is possible to design a single amiRNA to target multiple targets. The use of transcript database is recommended if the intended amiRNA is meant to be used for silencing transcripts present in the database. Additionally, use of filter on the database provides a mean for avoiding off-targets. For this, the input sequence, either as a gene ID or as a FASTA file, is termed as "foreground set" and serves to generate a set of 21-nt sequences. All the 21-nt sequences are compared against the transcript database, termed as the "background set," in case the "target specificity filter" is applied. All the 21-nt sequences from foreground set that match perfectly with sequences from background set between

Table 6.3 Transcript database available for designing of amiRNA in P-SAMS

Taxonomic category	Family	Genus
Green algae	Chlamydomonadaceae	*Chlamydomonas reinhardtii* (v5.5)
Dicot	Rosid/Brassicaceae	*Arabidopsis thaliana* (TAIR10)
Dicot	Rosid/Brassicaceae	*Camelina sativa* (v2)
Dicot	Rosid/Cucurbitaceae	*Cucurbita pepo* (v4.1)
Dicot	Rosid/Fabaceae	*Glycine max* (Wm82.a2.v1)
Dicot	Rosid/Fabaceae	*Vigna angularis* (v3)
Dicot	Rosid/Malvaceae	*Gossypium hirsutum* (NBI AD1 v1.1)
Dicot	Rosid/Rosaceae	*Malus domestica* (v1.0)
Dicot	Rosid/Euphorbiaceae	*Manihot esculenta* (v4, v6.1)
Dicot	Rosid/Salicaceae	*Populus trichocarpa* (v3.1)
Dicot	Rosid/Vitaceae	*Vitis vinifera* (Genoscape 12X)
Dicot	Asterid/Solanaceae	*Nicotiana benthamiana* (v1.0.1; v5; v5.1)
Dicot	Asterid/Solanaceae	*Nicotiana tabacum* (BX; K326; TN90)
Dicot	Asterid/Solanaceae	*Solanum tuberosum* (v3.4)
Dicot	Asterid/Solanaceae	*Solanum lycopersicum* (iTAG v2.3)
Dicot	Asterids/Apiaceae	*Daucus carota* (v2.0)
Monocot	Poaceae/Brachypodieae	*Brachypodium distachyon* (v1.2; v2.1)
Monocot	Poaceae/Pooideae	*Hordeum vulgare* (082214v1.30)
Monocot	Poaceae/Pooideae	*Triticum aestivum* (TGACv1)
Monocot	Poaceae/Oryzoideae	*Oryza sativa* (v7.0)
Monocot	Poaceae/Panicoideae	*Setaria italica* (v2.1)
Monocot	Poaceae/Panicoideae	*Setaria viridis* (v1)
Monocot	Poaceae/Panicoideae	*Sorghum bicolor* (v2.1; v3.1.1)
Monocot	Poaceae/Panicoideae	*Zea mays* (v6a)

positions 6-20 are discarded. This way, the filter minimizes off-target silencing by excluding undesired 21-nt candidates. The remaining candidates in the foreground set are grouped based on nucleotide matches at positions 1, 2, 3, and 21 and amiRNAs are predicted for each group. The amiRNA is designed such that the 5′ end is U, a G occupies at position 3 and corresponding to a C in the target. Further, the 21st position is intentionally mismatched. Once the tool has predicted amiRNAs, "TargetFinder" tool is employed (Allen et al. 2005; Fahlgren et al. 2007; Fahlgren and Carrington 2010) to identify targets from the specified database. Three optimal amiRNA candidates are suggested per prediction. The tool finally generates the primers required for synthesis and engineering of amiRNA in the *MIR390* precursor of either *A. thaliana* or *O. sativa* (Fig. 6.2a). A detailed step-by-step experimental guide is provided both at the weblink and also in the publication (Carbonell 2019).

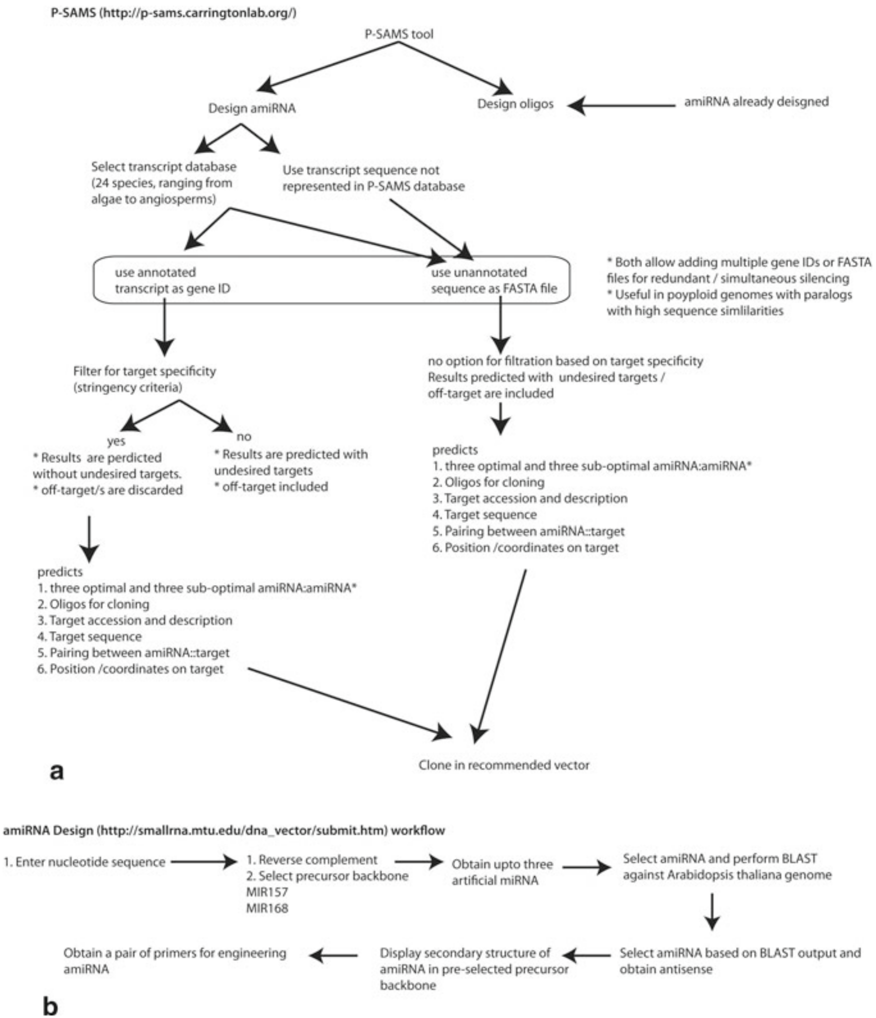

Fig. 6.2 Schematic representation of workflow of P-SAMS (**a**) and amiRNA Design (**b**) tool. In both P-SAMS (**a**) and amiRNA Design (**b**), multiple steps are implemented in a specific and sequential order. In this process, results from one step generate the input data for the next step. The various steps are implemented in a unidirectional manner (solid arrows)

6.2.3 amiRNA Design/MicroRNA Designer

This is yet another amiRNA designer tool, developed in Prof. Guiliang Tang's lab, which permits limited user-defined criteria (http://smallrna.mtu.edu/dna_vector/submit.htm). The webpages for design of amiRNA are sequential, and begin with the user entering a nucleotide sequence against which amiRNA is to be designed. Relative to other tools, here, the user does not have the option to select any existing database

to identify close homologs, or gene IDs. Once the target sequence is submitted, the program generates its reverse complement and prompts the user to select *MIR157* or *MIR168* precursor. Up to three amiRNAs are predicted without information about the hybridization energy, position, and mismatch information, and the user can then perform BLAST against the *Arabidopsis thaliana* genome to identify potential off-targets. Once a desired amiRNA is selected, the user can design primer sets for engineering in the pre-selected precursor backbone (Fig. 6.2b).

6.2.4 amiRNA Designer

This new tool for amiRNA design is available for download and used as a local installation (http://www.cs.put.poznan.pl/arybarczyk/AmiRNA/; Mickiewicz et al. 2016). It allows users to customize and alter the design parameters depending on the requirement. The authors state that experimental validation of amiRNA designed by other tools such as WMD3, often times fail to achieve optimal silencing efficiencies, as these use limited parameters for prediction (Li et al. 2013; Mickiewicz et al. 2016). Keeping in mind the drawbacks associated with other tools, the authors created a sophisticated amiRNA Designer program that compares free energy decomposition profile of the amiRNA-target to those of known miRNA/miRNA* and miRNA targets. The authors claim that the predictions based on the newly developed tool are more accurate and amiRNAs demonstate greater silencing efficiencies. The amiRNA Designer is a semi-automated program that allows researchers to use a preset design parameters. Alternatively, the user may modify the parameters using perl scripts available at the download site. The program first requires users to load data under the "Perspective" tab using a defined "InputDescriptor" format. The InputDescriptor contains datasets of miRNA including sequence of precursor and mature miRNA, coordinates, miRNA ID, target dataset as separate input files. These are required to generate thermodynamic profiles of miRNA-target interactions. The input file is processed to generate secondary structures for both miRNA-miRNA* precursor and miRNA-target, with respective thermodynamic profiles. The thermodynamic profiles as input data (as text, csv, or Excel file) is subsequently used by "Design" tool along with target gene sequence data. After this step, the Design tool selects appropriate target site and predicts amiRNA. The next few steps act as filters and the program predicts and analyses the secondary structure of amiRNA-target, the free energy of hybridization, and compares with the data generated during the "input" stage of miRNA-target, to create an optimal amiRNA list. The user can choose to alter design criteria and parameters including relaxing the stringency, introducing mismatches, or base substitution in the amiRNAs (Mickiewicz et al. 2016).

6.3 Artificial miRNA-Mediated Gene Silencing: Case Studies on Functional Genomics and Trait Modification

amiRNA-mediated gene silencing is a preferred strategy primarily owing to specificity rendered for preventing off-target silencing. Using amiRNA platform, it is now possible to knock-down genes in virtually any plant system where a reasonably high level of genome-wide transcript information is available. Opportunities now exist for functional genomics and introduction of novel traits even in non-model organisms such as *Cenchrus ciliaris*, *Centaurea*, *Cichorium*, *Eschscholzia*, *Gerbera*, *Gingko*, *Ipomoea*, *Leymus*, *Micromonas*. Especially in context to crops, flexible format of amiRNA design permits determining role of a single gene or members of entire gene families. It is also possible to dissect functions of alleles, paralogous and homoelogous copies and even splice variants (Fig. 6.3). Further, unraveling functional divergence among closely related genes as well divergent orthologous genes

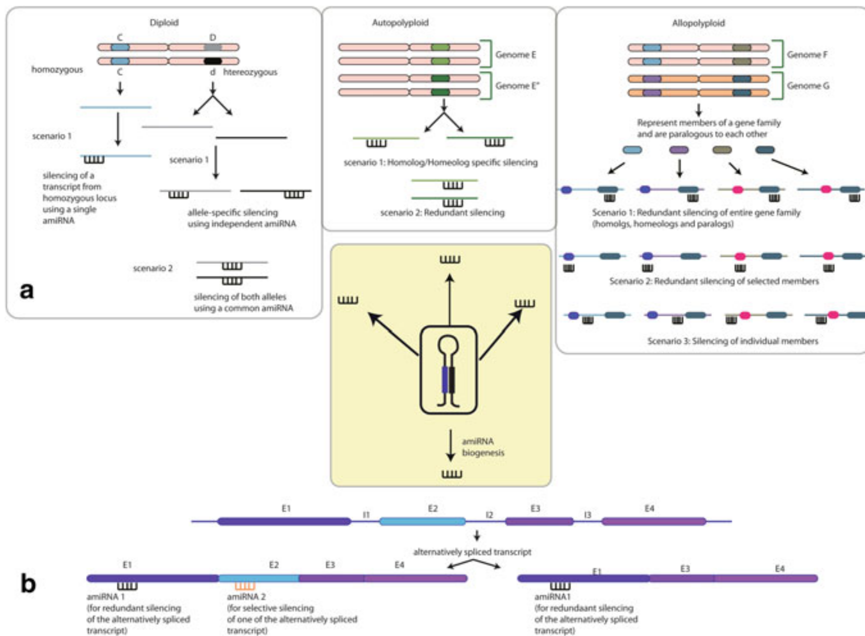

Fig. 6.3 Artificial miRNA technology is based on highly specific complementary base-pairing between a 21-nt small RNA and target mRNA. This requirement of precise base-pairing can be used to silence any desired sequence such as alleles, homeologs, paralogs (**a**), or splice forms (**b**) present in diploid or polyploid genomes. The alleles, homeologs, and paralogs are indicated with slightly different shade of the color indicating sequence variation. In panel 3A, various silencing scenarios are contextualized to diploid, auto-, and allo-polyploid in interest of clarity. However, these scenarios are not exclusive to any ploidy level. Careful designing of amiRNAs against unique sequences permit selective silencing of candidate transcript(s) originating from a single locus. AmiRNAs designed against shared or conserved sequences also allow multiple loci to be silenced in a redundant manner

is now tenable. All these aspects qualify amiRNA technology as a powerful tool for analysing gene functions in polyploid crops. Even diploid crops harboring multiple gene copies are likely to benefit from amiRNA technology. Furthermore, the miRNAs can be regulated by a range of natural and synthetic promoters that are available for precise regulation of candidate targets in a tissue-specific, or inducible manner. As the technology matures, novel and unique modifications will increase the spectrum of applications. In the following section, case studies are described to illustrate the adoption of this technology in plant species (Table 6.4).

6.3.1 Gene Silencing in Polyploid System: Brassica Species as Case Study

Brassica species constitute a major source of oil-seed and vegetable worldwide (Rakow 2004; Warwick et al. 2019). Genome-wide analysis of *Brassica* species reveals a unique genome architecture suggesting an interesting evolutionary history. A sub-genome structure harboring multiple gene copies is evident not only in *Brassica* species but also other members of Brassicaceae. Evolutionary biologists hypothesize that recurrent whole genome duplication in common ancestors resulted in establishment of multiple gene copies. The multiple gene copies within polyploid genomes are termed as "homeologs" which are reported to undergo divergence at both sequence and functional level. The outcome of gene diversification can be manifold. Gene homeologs may be either functionally redundant or acquire novel functions, a phenomenon commonly known as neo-functionalization. Alternatively, a homeolog may lose its function and persist in the genomes as pseudogenes. All these aspects make functional characterization of gene homeologs important but also challenging (Lysak et al. 2005, 2007; Schranz et al. 2006; Wang et al. 2011; Cheng et al. 2018; Liu et al. 2014; Lee et al. 2020). amiRNAs have enabled dissection of gene function in Brassicas. The following account summarizes representative case studies.

amiRNA design for redundant silencing of *SHP1* and *SHP2*: In one of the first reported case study, the potential of amiRNAs for simultaneous down-regulation of paralogs was tested. In *A. thaliana*, *SHATTERPROOF1 (SHP1)* and *SHATTERPROOF2* (*SHP2*) are involved in fruit dehiscence. Since spontaneous pod-shattering causes yield losses, silencing of *SHP1* and *SHP2* promises shatter resistance (Liljegren et al. 2000). The authors surveyed natural variation in *SHP1* and *SHP2* homolgs across *Brassica* species. Two amiRNAs were designed with differing hybridization energies and predicted for differing cleavage efficiency (Dhakate et al. 2014). In this study, the potential of redundant silencing of paralogous genes was demonstrated through a transient assay system in *N. benthamiana*. Co-infiltration of either of the amiRNAs (amiRNA-SHP1 or amiRNA-SHP2) with *SHP1* or *SHP2* from *B. napus* (AACC; allotetraploid), *B. juncea* (AABB; allotetraploid), and *A. thaliana* (diploid) revealed that amiRNA-*SHP2* could redundantly direct the cleavage of the paralogous copies of *SHP1* and *SHP2* (Dhakate et al. 2014).

Table 6.4 Representative case studies for use of artificial miRNA technology for functional genomics and trait manipulation

S. No.	Trait	Remarks	Reference
Chlamydomonas reinhardtii			
1	Inducible gene expression system	Light-induced amiRNA biogenesis to control hydrogen production (optogenic regulation)	Wang, Jiang, et al. (2017)
2	Fatty acid metabolism	amiRNA mediated downregulation of stearoyl-ACP desaturase to increase stearic acid levels	de Jaeger et al. (2017)
3	Fatty acid production	amiRNA against PEP carboxylase leads to increased fatty acid production	Wang, Chen, et al. (2017)
4	Gene function analysis	Establishment of amiRNA technology in *Chalamydomonas*	Molnar et al. (2009), Hu et al. (2014)
5	Thermo-tolerance	Role of HSF1in conferring thermo-tolerance demonstrated by amiRNA against *C. reinhardtii* HSF1	Schmollinger et al. (2010)
Physcomitrella patens			
6	Functional genomics	Demonstration of gene function analysis through amiRNAs targeting *PpFtsZ2-1* (involved in chloroplast division), *PpGNT1* encoding an N-acetylglucosaminyltransferase	Khraiwesh et al. (2008, 2011), Fattash et al. (2012)
Marchantia polymorpha			
7	Gene function analysis	Use of amiRNA demonstrated cleavage of Auxin Response Factor (*MpARF1*), Response regulator (*Mp-RR-B*) and Enhancer of Zeste [*MpE(z)*]	Flores-Sandoval et al. (2016)

(continued)

Table 6.4 (continued)

S. No.	Trait	Remarks	Reference
Arabidopsis thaliana (diploid)			
8	Seed oil composition	amiRNA against delta-12-desaturase (*FAD2*), Fatty acid elongase (*FAE1*), and Fatty acyl-ACP thioesterase B (*FATB*)	Belide et al. (2012)
9	Demonstration of methods for differentiating the *in-vivo* effects of *FT* transcripts and protein molecules	Application of artificial miRNAs to generate evidence for necessity of *FT* mRNA is in phloem companion cells for floral induction	Mathieu et al. (2007)
10	Pollen development	amiRNA driven by pollen-specific promoter to study small RNA processing in pollen grains	Grant-Downton et al. (2013)
11	Gene function analysis	Generation of amiRNA library for functional genomics of gene and gene family members through specific and redundant silencing; *In-silico* prediction of more than 533,000 amiRNA targeting ca. 27,000 genes	Hauser et al. (2013; 2019), Zhang et al. (2018)
12	Virus resistance	Combination of two amiRNAs-amiRNA-HcPro and amiRNA-TuCP against two highly conserved region to engineer resistance against Turnip mosaic virus	Lafforgue et al. (2013)
13	Virus resistance	Separate amiRNAs against viral suppresors P69 of turnip yellow mosaic virus (TYMV) and HC-Pro of turnip mosaic virus (TuMV); plant expressing the two amiRNAs confer resistance against both viruses	Niu et al. (2006)
14	miRNA function	Artificial miRNA system to understand role of environment and developmental events in miRNA mediated gene regulation	von Born et al. (2018)
15	Plant development under heat stress	amiRNA against miR160 to study effect of heat stress on development	Lin et al. (2018)
16	Disease resistance (powdery mildew)	amiRNA mediated silencing of *XCT* leads to reduced resistance against powdery mildew	Xu et al. (2017)

(continued)

Table 6.4 (continued)

S. No.	Trait	Remarks	Reference
17	Virus resistance	amiRNA to explore role of plant cytidine deaminases in virus resistance	Martín et al. (2017)
18	Photomorphogenesis	amiRNA mediated silencing of *PPK* reveals their role in blue-light dependent phosphorylation of*CRY2*	Liu et al. (2017)
19	miRNA function	Artificial miRNA targeted against stemloop region or mature miRNA can be used for silencing of individual member or all members of endogenous miRNA gene family. The authors also suggest that target cleavage also occurs in the nucleus	Eamens et al. (2011)
20	Target cleavage efficiency of miRNA	Sequence complementarities determine target cleavage efficiency was examined by silencing of *MYB33/65* by comparing efficiencies of miR159 and amiRNA against *MYB33/65*	Deveson et al. (2013)
21	Autoimmunity	Artificial miRNA mediated downregulation of Suppressor of CHS1-2 (SOC) homolog *SOC3* to understand it role in low temperature-dependent autoimmune response	Zhang et al. (2017)
22	Pollen development	AmiRNA based knockdown of the paralogs *NIP4;1* and *NIP4;2* causes defect in pollen germination and tube length	Di Giorgio et al. (2016)
Camellina sativa (polyploid)			
23	Fatty acid profile	Expression of amiRNA against Fatty acyl thioesterase (*FATB*) in a seed-specific manner alters fatty acid profile	Ozseyhan et al. (2018)
24	Fatty acid composition	Downregulation of *PDAT* and *DGAT1* through amiRNA alters levels of 18:3n-3 levels in seeds	Marmon et al. (2017)

(continued)

Table 6.4 (continued)

S. No.	Trait	Remarks	Reference
Oryza sativa			
25	Panicle exsertion	amiRNA against endogenous *Eui1* gene and target mimicry against amiRNA	Chen et al. (2013)
26		Sequestration of *OsmiR396* by target mimicry and over-expression of *OsGRF8* causes resistance against brown plant hopper insect	Dai et al. (2019)
27	Gene function analysis	amiRNA against Pectin-lyase-like (*OsPLL3* and *OsPLL4*) to decipher gene function	Zheng et al. (2018)
28	Starch characteristic	Starch characteristics and digestibility through downregulation of *starch branching enzyme IIb, SBEIIb*	Butardo et al. (2011)
30	Starch biosynthesis	Artificial miRNA mediated silencing of starch binding domain-containing protein (*SBDCP1*) led to starch with lower amylose content and increased proportion of amylopectin chain with a higher level of polymerization	Cakir et al. (2019)
31	Aluminium responsiveness	*ASR1* and *ASR5* act together to regulate Aluminium responsiveness	Arenhart et al. (2016)
32	Phytic acid level	Reduction in phytic acid level by amiRNA mediated downregulation of *OsMRP5* driven by seed specific Ole18 promoter	Li et al. (2014b)
Wheat			
33	Resistance against Wheat streak mosaic virus	Five amiRNAs designed against Wheat streak mosaic virus (and engineered into synthetic polycistronic miR395 backbone to confer virus resistance	Fahim et al. (2012)
34	Grain hardiness	amiRNA against Puroindoline a (Pina) and Puroindoline b (Pinb) in wheat and their orthologues Secaloindoline a (Sina) and Secaloindoline b (Sinb) genes in triticale	Gasparis et al. (2017)

(continued)

Table 6.4 (continued)

S. No.	Trait	Remarks	Reference
Malus domestica			
35	Gene function analysis	amiRNA against phytoene desaturase to understand gene function	Charrier et al. (2019)
Nicotiana tabacum			
36	Virus resistance	Nine amiRNA against potato virus Y; plants expressing amiRNA against NIb and CP gene exhibited higher level of tolerance	Song et al. (2014)
37	Virus resistance	Polycistronic backbone based on *Hordeum vulgare MIR171* precusor to engineer amiRNA against wheat dwarf virus;	Kis et al. (2016)
38	Insect resistance	amiR-24 against Chitinase gene confers tolerance against *Helicoverpa armigera*	Agrawal et al. (2015)
39	Aphid resistance	acetylcholinesterase 2 (*MpAChE2*) gene of *Myzus persicae* was targeted by production of amiRNA in plants to obtain aphid resistance	Guo, Song, et al. (2014)
Nicotiana benthamiana			
40	Virus resistance (tomato spotted wilt visus)	Use of *Nicotiana benthamiana* pathosystem to identify artificial miRNAs against tomato spotted wilt virus	Carbonell et al. (2019)
41	Viroid infection	amiRNA together with synthetic tasiRNA to study viroid infection and spread using Potato spindle tuber viroid (PSTVd)–*Nicotiana benthamiana* pathosystem	Carbonell and Daròs (2017)
42	Virus resistance	three amiRNAs -amiR1-CP, amiR4-MP, and amiR6-Rep targeting cucumber green mottle mosaic virus	Liang et al. (2019)
43	amiRNA silencing mechanism	Use of amiRNA in transient assay system in *N. benthamiana*, and comparing with stable transgenic lines of *A. thaliana* to understand translational repression also as a mode of action of amiRNA	Yu and Pilot (2014)

(continued)

Table 6.4 (continued)

S. No.	Trait	Remarks	Reference
Brassica juncea (allotetraploid) and *Brassica napus* (allotetraploid)			
44	Pod shattering	Redundant silencing of *SHP1* and *SHP2*	Dhakate et al. (2014)
45	Flowering time	Silencing of *FT* homologs from *B. napus* and *B. juncea*	Tyagi et al. (2018)
46	Flowering time	Silencing of *SOC1* from *B. juncea*	Tyagi et al. (2019)
47	Fatty acid profile	Redundant silencing of *SAD*	Sun et al. (2013)
48	Sinapine content	Redundant silencing of SCT and SGT to reduce seed sinapine content	Kajla et al. (2017)
49	Development and immunity	Investigating the role of miR1885-dependent phasiRNA, phasi-130-4 by cloning phasiRNA as amiRNA to silence photosynthetic related gene *BrCP24*	Cui et al. (2020)
Brachypodium distachyon			
49	Flowering time	amiRNA mediated silencing of *VRN1* or *AG* in flowering time control	Lomax et al. (2018)
50	Flowering time	Silencing of a splice variant of *FT* through amiRNA causes early flowering	Qin et al. (2017)
Solanum lycopersicon			
51	Virus resistance	Synthetic tasi-RNA to confer resistance against Tomato spotted wilt virus	Carbonell et al. (2019)
52	Virus resistance	Redundant silencing of genes *2a* and *2b* leads to resistance against *Cucumber mosaic virus (CMV)*	Zhang et al. (2011)
53	Glutamate content	Role of NADH-dependent glutamate dehydrogenase in regulating glutamate content in fruit	Ferraro et al. (2015)
Solanum melongena			

(continued)

Table 6.4 (continued)

S. No.	Trait	Remarks	Reference
54	Male sterility	Silencing of *SmTAF10* and *SmTAF13* to confer male sterility. An inducible system with amiRNA resistant forms of *SmTAF10* and *SmTAF13* restores fertility	Toppino et al. (2011)
Solanum tuberosum			
55	Gene function	Development of amiRNA technology and demonstration of proof-of-concept by silencing of *CBP80/ABH1* gene to confer drought tolerance	Wyrzykowska et al. (2016)
56	Oxidative browning	amiRNA mediated silencing of Polyphenol oxidase (*PPO*) reduces oxidative browning in potatoes	Chi et al. (2016)
Glycine max Soybean			
57	Seed size	amiRNA against *BS1* gene to regulate seed size	Ge et al. (2016)
58	Gene function analysis	Silencing of *GmWRP1*, a novel WRKY-related protein showed its role in senescence and nodulation	Wang et al. (2016)
Medicago truncatula			
59	Pi-transport and mycorrhizal symbiosis	Silencing of H + -ATPase gene *HA1* through amiRNA leads to reduced arbuscular mycorrhiza association and impaired phosphate uptake	Krajinski et al. (2014)
60	Mycorrhizal association	Role of *MtErf1* in mycorrizal association	Devers et al. (2013)
Withania somnifera (L)			
61	Secondary metabolism	Role of *MYC2* in Withanolide and phytosterol biosynthesis	Sharma et al. (2019)
Vitis vinifera			
62	Functional genomics	Development of amiRNA technology for functional genomics; proof-of-concept using amiRNA against *GFP* using *Vitis vinifera* miR319e backbone in *N. benthamiana*	Castro et al. (2016)

(continued)

Table 6.4 (continued)

S. No.	Trait	Remarks	Reference
Manihot esculenta (Cassava)			
63	Virus resistance	Ten amiRNAs against Cassava brown streak virus (CBSV) and Ugandan cassava brown streak virus (UCBSV)- P1(CBSV and UCBSV), P3 (CBSV and UCBSV), CI (UCBSV), Nib (CBSV and UCBSV), CP (UCBSV) and the untranslated region (3′-UTR) tested in N. benthamiana. Resistance obtained against CBSV in stable lines with amiRNA against P1 and Nlb; and against UCBSV in lines with amiRNA against *P1* and *CP* genes	Wagaba et al. (2016)
Petunia hybrid			
64	amiRNA biogenesis and processing	Use of amiRNA against Chalcone synthase (*CHS*) to show that other, unwanted small RNAs are produced from the amiRNA precursor and these could cause off-target silencing	Guo, Han, et al. (2014)
Populus trichocarpa			
65	Gene function analysis	Utilization of amiRNA technology in forest tree species demonstrated by downregulation of members of phenylalanine ammonia-lyase (*PAL*) family; specific targeting of *PAL2*, *PAL4*, and *PAL5* causes increase in transcript abundance of other *PAL* family members-*PAL1* and *PAL3*	Shi et al. (2010)
Lemna minor			
66	Gene function	Development of amiRNA technology and demonstration by silencing of *CH42*, a Magnesium chelatase that leads to reduced chlorophyll pigmentation	Cantó-Pastor et al. (2015)
Multiple plants (*Arabidopsis thaliana, Nicotiana benthamiana, Solanum lycopersicum, Helianthus annuus, Catharanthus roseus, Zea mays, and Oryza sativa*			
68	Parallel evaluation of amiRNA efficacy, fate of target mRNA, and translation product	Epitope-tagged protein-based amiRNA (*ETP*amir) screens	Li et al. (2013)

(continued)

Table 6.4 (continued)

S. No.	Trait	Remarks	Reference
Others			
69	Artificial miRNA mediated gene silencing efficiency	Presence of asymmetric bulge was shown to increase silencing efficacy and enhance resistance against tomato bush stunt virus	Zhang et al. (2020a)
70	Gene function for stress tolerance	Designing of amiRNA for biotic and abiotic stress tolerance in *Camellia sinensis*	Jeyaraj et al. (2020)
71	Virus resistance	Predicting miRNA and amiRNA for Sugarcane Bacilliform Guadeloupe A Virus resistance in sugarcane (*Saccharum officinarum* L.)	Ashraf et al. (2020)

Functional characterization of *FLOWERING LOCUS T*: Implementing a complementary approach of over-expression and amiRNA-meditated silencing, the researchers investigated the function of *FT* homologs in the genetic background of *B.juncea* var. Varuna and *ft-10* mutant in *A. thaliana.* A single amiRNA was designed against *FT* from *B. juncea* and engineered in the backbone of *A. thaliana MIR319a* under the control of *CaMV35S* promoter. The amiRNA was designed to redundantly silence multiple *FT* homeologs present in the *B. juncea* genome (Tyagi et al. 2018). The applicability of FT-amiRNA in targeting *FT* transcripts from as many as 17 cultivars of six *Brassica* species was predicted. This was based on sequence complementarity and thermodynamics of hybridization. A significant correlation was observed between the transcript levels of FT-amiRNA and the extent of target cleavage in the transgenic lines of *B. juncea.* Silencing of *FT* through amiRNA led to severely delayed flowering, rudimentary siliques with non-viable seeds, small and constricted stomata on leaves, revealing multiple roles of *FT* homeologs during plant development (Tyagi et al. 2018). The study suggested the potential of deploying designed amiRNA in a wider germplasm of *Brassica.*

Role of *SUPPRESSOR OF OVEREXPRESSION OF CONSTANS1*: Flowering time control is a key life-history trait and the role of *SOC1* in model plant, *A. thaliana* is well known. However, at the time of the study, detailed characterization of *SOC1* from polyploid *Brassica* species was not examined; neither additional roles of *SOC1* were deciphered. Tyagi et al. (2019) designed amiRNA for analysing the *Brassica SOC1* function. Withdrawal of *B. juncea SOC1* function was achieved by predicting amiRNA against *Brassica SOC1.* Analysis of the transgenic lines revealed successful cleavage of *Brassica SOC1* transcripts. Sequence characterization of the cleaved products of *B. juncea SOC1* were found to map to the two progenitor genomes of the allotetraploid *B. juncea.* Phenotyping of the SOC1-amiRNA lines showed delayed flowering in *B. juncea* by up to 13 days. Delayed flowering was accompanied by altered fatty acid profile in developing seeds including levels of saturated fatty acid (SFA), and unsaturated fatty acids. Among other traits, redundant silencing of *Brassica SOC1* resulted in reduced number of lateral branches, number of siliques on main and lateral branches, seed weight, and seed yield revealing additional roles of *SOC1* conferred, plausibly by distinct homeologs, during development (Tyagi et al. 2019).

Altered fatty acid profile: Enhancement in saturated fatty acid content in *B. napus* was achieved by simultaneous up-regulation of fatty acyl-ACP thioesterase B (*BnFatB*) and down-regulation of stearoyl-ACP desaturases (*BnSAD*s). *B. napus* genome contains eight copies of *BnSAD*s- *BnaA-SAD1*, *BnaA-SAD2*, *BnaC-SAD1*, *BnaC-SAD2*, *BnDES5a*, *BnDES5b*, *BnDES5c*, and *BnDES5d*. In order to achieve redundant silencing of all the eight copies, two amiRNAs were predicted. While the first amiRNA was designed to target *BnDES5a*, *BnDES5b*, *BnDES5c*, and *BnDES5d* (BnSADamiR1), the second one targeted *BnaA-SAD1*, *BnaA-SAD2*, *BnaC-SAD1*, and *BnaC-SAD2* (BnSADamiR2). A single construct containing the two amiRNAs, along with BnFatB cassette was generated. All the cassettes were under the control of seed-specific napin promoter and used for *B. napus* transformation. Stemloop PCR analysis of transgenic seeds showed high levels of the two amiRNAs with

reduced levels of all the eight copies of *BnSADs* implying that the amiRNAs were able to redundantly silence homeologous and paralogous copies of stearoyl-ACP desaturases present in the polyploid *B. napus* genome. Fatty acid content and profile analysis showed that the total saturated fatty acid content increased by up to 45% in transgenic plants. The triacylglycerol content was similarly altered. Finally, the increase in saturated fatty acid was also correlated to increase in melting temperature from −10 °C in untransformed plants to +15 °C in transgenic lines with high SFA (Sun et al. 2013).

Seed sinapine content manipulation: Reduced seed sinapine content is a desirable trait with recommended levels of < 3.0 mg/g of dry seed weight (DSW) for animals and < 1.0 mg/g DSW for humans. Two genes, *SGT* encoding UDP-glucose: sinapate glucosyltransferase and *SCT* encoding sinapoylglucose:choline sinapoyltransferase, are required for sinapine biosynthesis. In order to achieve the recommended levels in *B. juncea*, the authors first isolated four paralogs of *SGT* and two paralogs of *SCT* from *B. juncea.* Three parallel RNAi-based strategies including amiRNA were devised. Transcriptional regulation of the RNAi constructs were under the control of napin promoter for *SGT*, or native promoter for *SCT*. The amiRNAs were able to silence the paralogous copies of *SGT*, and *SCT*. Analysis of sinapine content in seeds of transgenic lines of *B. juncea* obtained with the three approaches revealed 11% reduction using amiRNA (Kajla et al. 2017).

Disease resistance and immunity: The role of a miRNA dependent phasiRNA was investigated by generating an amiRNA using the phasiRNA for functional characterization. The miRNA, miR1885 was found to directly silence a resistance gene *BraTNL1*, a member of TIR-NBS-LRR class of disease resistance genes. A phasiRNA, phasiR130-4 is produced in a miR1885-dependent manner in *B. napus* through trans-acting small interfering RNAs (ta-siRNAs)-mediated silencing employing the Trans-Acting Silencing (TAS) gene *BraTIR1* during Turnip mosaic virus (TuMV) infection. The miR1885-dependent phasiRNA targets photosynthesis-related gene BraCP24. In order to understand the role, an amiRNA, amiR130-4 was created by cloning the phasiRNA, phasiR130-4 in the *AthMIR159* backbone. The amiR130-4 was transiently coexpressed with wild-type or mutated *BraCP24* (*BraCP24m*) in *N. benthamiana* to confirm interaction. The dual role of miR1885 in modulating development and immunity was established (Cui et al. 2020).

6.3.2 Functional Genomics

The following section presents case examples wherein amiRNA technology has been used for unraveling gene function in diverse species.

amiRNA library for functional genomics in *A. thaliana*: Computationally derived miRNAs were predicted for 18117 loci with 22000 target classes in the *A. thaliana* genome and family-specific amiRNAs were designed. Based on computational prediction, 22000 amiRNAs were synthesized as sub-libraries, with each library containing between 1500–4000 amiRNAs. The amiRNAs with a potential to

silence up to 16 genes were selected based on defined parameters including up to five mismatches in a gap-less alignment; one hit per gene; no off-targets; minimum two targets, as implemented in WMD tool (Hauser et al. 2013, 2019). Libraries specific to genes with specific functional classes include those targeting protein kinases, phosphatases, transporter proteins, zinc finger proteins, MAPKKKs, unknown proteins were characterized. Functional analysis of transgenic plants with amiRNA against zinc finger homeodomain and MAPKKK exhibited novel phenotypes. Phenotypes of transgenic plants with amiRNAs against phosphatase families recapitulated those reported earlier for loss-of-function mutants (Hauser et al. 2013, 2019). The amiRNA library is available from ABRC as an amiRNA pool (sub-libraries) and as T3 generation seeds of the transgenic plants and constitutes a valuable resource for functional genomics analysis (Hauser et al. 2013, 2019).

Multiplex gene silencing for functional genomics in *A. thaliana* and *Oryza*: A database of nearly 533400 amiRNAs has been developed for silencing of about 27130 genes of the *A. thaliana.* In addition to designing amiRNAs achieving a near-saturation coverage (98.9%) of gene space, the authors also used tRNA-miRNA tandem repeats for devising a multiplexed gene silencing strategy. A novel rapid screening methodology was developed with an amiRNA engineered within an intron in the *GFP* reporter. Co-transcription of the reporter and the amiRNA allowed the monitoring of the reporter as a proxy for amiRNA biogenesis and function, which was also validated by testing the correlation between reporter activity and amiRNA-mediated gene silencing. As a proof-of-concept, amiRNAs that could redundantly silence multiple genes (*GLK1-GLK2*; *APK2A-APK2B*; *SERK1-SERK2-SERK3-SERK4-SERK5*) were characterized. In addition, amiRNAs that specifically target splice variants, *SR45.1* and *SR45.2*, involved in petal and root development, respectively, were used. Finally, a tRNA-pre-miRNA system (tRNA^{Gly}-premiR319a) was developed that allowed co-transcription of five different amiRNAs (*FLS2, EFR, CERK1, PEPR1,* and *RLP23*) and silencing of the target candidate transcripts (Zhang et al. 2018).

6.4 Advances in AmiRNA Technology

The amiRNA technology has now emerged as a powerful tool to dissect gene function and for trait modification. Several researchers have successfully modified or adapted amiRNA technology for various applications and illustrative examples are discussed in the following section.

Chimeric amiRNA backbone: The idea of engineering a synthetic MIRNA precursor backbone that can efficiently process a mature miRNA was conceptualized. The natural precursor sequence of *MIRNA* genes is divided into three distinct zones, a basal stem, the stem containing miRNA/miRNA*, and the distal loop. An analysis of precursor length and secondary structures of *MIRNA* genes among various species revealed that the distal stem-loop of *MIR390a* from *A. thaliana* and *MIR390* of *O. sativa* is among the shortest. It was speculated that this feature

may contribute significantly to cost-effectiveness. The pre-Ath-*miR390a* is known to express high levels of amiRNA in dicots (Carbonell et al. 2014). Given the short size of *MIR390* precursor from *O. sativa*, and high-expressivity of Ath-MIR390a, a chimeric precursor backbone for monocots was designed. The applicability was then examined in *B. distachyon.* Levels of mature miRNA and amiRNA from the chimeric precursor were compared with unmodified precursor (*OsMIR390).* Highest level of amiRNA accumulation was observed when the basal stem was derived from *O. sativa* and the distal loop from *A. thaliana.* Analysis of amiRNA population in these transgenic lines revealed that the possibility of off-target silencing was higher when unmodified precursor of *OsMIR390* was used (Carbonell et al. 2015).

Synthetic polycistronic amiRNA backbone: The potential of amiRNA technology can be further expanded if amiRNAs against multiple genes can be delivered simultaneously. In order to achieve this, a polycistronic amiRNA backbone was assembled wherein miRNA precursor (Ath-miR319a) and tRNA ($tRNA^{Gly}$) sequences are tandemly arranged (Fig. 6.4; Zhang et al. 2018). The researchers validated a construct design in which five different amiRNAs were engineered.

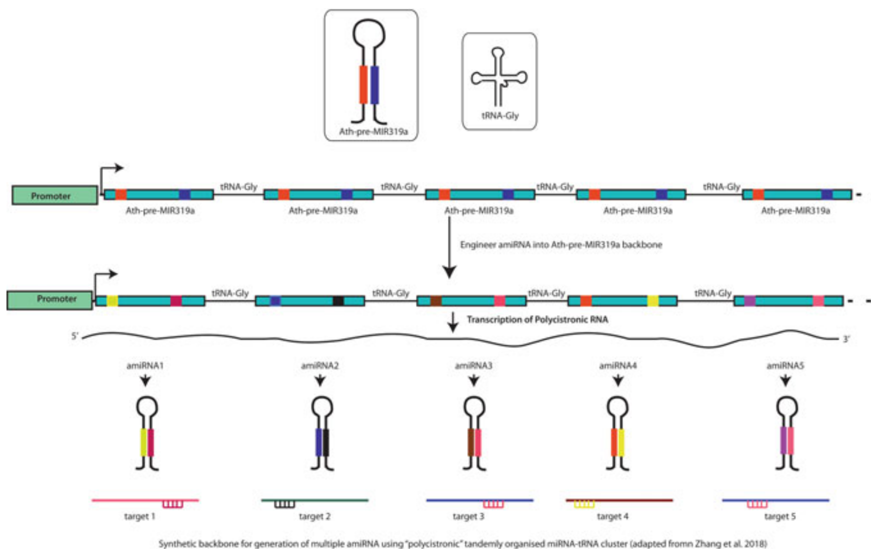

Fig. 6.4 Digrammatic representation of a synthetic polycistronic amiRNA delivery backbone. Multiple copies of naturally occurring precursor sequences of *MIR319a* and $tRNA^{Gly}$ are alternately arranged and fused end-to-end to create a synthetic polycistronic backbone. Each of the precursor can be engineered to replace the native miR319/miR319* with amiRNA/amiRNA*. The synthetic pre-MIR319a-tRNA backbone is driven by a strong promoter to transcribe a polycistronic RNA. The transcript mimics a primary miRNA with multiple hairpin loops of tRNA and miRNA. The polycistronic RNA is processed to generate independent precursors with amiRNA which can simultaneously silence different targets (Zhang et al. 2018). The sequences corresponding to native miR319 and miR319* are indicated by red and blue boxes. In the present example, different amiRNA/amiRNA* are denoted by different pairs of colored boxes

The polycistronic construct harboring five amiRNAs, and targeting five different immune receptor genes - *FLS2*, *EFR*, *CERK1*, *PEPR1*, and *RLP23,* was introduced into *A. thaliana.* The transgenic lines showed down-regulation of the respective targets (Zhang et al. 2018). Such polycistronic amiRNA delivery vector systems based on *Hordeum vulgare MIR171* backbone have been used against wheat dwarf virus to confer tolerance (Kis et al. 2016).

Trans-acting small interfering RNA-based MicroRNA-induced Gene Silencing (MIGS): ta-siRNAs are generated when TAS-transcripts are cleaved by miR173. Further processing of these cleaved TAS-transcripts via a combination of DCL4 and RDR6 activity lead to biogenesis of siRNAs which further contribute to gene silencing (de Felippes and Weigel 2009; de Felippes et al. 2012; Jacobs et al. 2016). This aspect of tasi-RNA has been exploited to trigger potent silencing of target genes. Toward this goal, the candidate gene to be silenced is fused with a miR173 target sequence derived from a TAS transcript. Such a chimeric transcript is then introduced into the plant as a transgene. The chimeric transgene containing target site for miR173 is cleaved to produce 21-nt small RNAs by DCL4. Following this, the small RNAs guide silencing of the endogenous unmodified copy of the gene. This process has been referred to as MicroRNA-induced Gene Silencing (de Felippes et al. 2012). Although amiRNAs are not involved in this strategy, the tasi-RNA pathway is cleverly leveraged to elicit siRNA production against any locus of choice. Since miR173 is present only in *A. thaliana* and its close relatives, MIGS can be applied in other species by co-expressing *MIR173* along with target gene (Han et al. 2015).

Protoplasts as transient system for protein-based amiRNA screening method: A protoplast-based method was developed for quick screening of not only amiRNA efficiency and mode of action, but also quantification and accumulation of target proteins (Li et al. 2013, 2014a; Zhang et al. 2017). In this method, the target mRNA is conjugated with Hemagglutinin (HA) as an epitope. The epitope levels can be rapidly quantified through immunoblotting and densitometric scans using anti-HA antibodies. When the HA-tagged target mRNA is co-infiltrated with amiRNA, the amount of the HA serves to reflect the silencing efficiency of the amiRNA. The authors validated and provide a proof-of-concept by using HA-tagged target mRNA to a variety of *A. thaliana* genes including *MEKK1* (*MEK kinase*), *PDS3* (*phytoene desaturase*), *NPK1-related Protein Kinase1* (*ANP1*), *ANP2, ANP3, MAPKKK17*, *MAPKKK18*, *LysM Domain GPI-anchored Protein2* (*LYM2*), and *Zinc Finger of Arabidopsis thaliana6* (*ZAT6*). HA-tagged transcripts were co-infiltrated in mesophyll protoplasts along with amiRNAs of differing potency and the level of HA was determined by antibody. The data was further validated by generating stable transgenic lines with the candidate amiRNAs and phenotyped to support the transient assay results (Li et al. 2013, 2014a; Zhang et al. 2017). The tool was also tested for redundant silencing of paralogous members of gene families such as *RECEPTOR FOR ACTIVATED C KINASE1 (RACK1*) family and the *MAPKKK YDA* family (*ALPHA, YDA,* and *RACK1a, RACK1bGAMMA*) members by using appropriately designed amiRNAs. Finally, the authors combined a polycistronic precursor-based amiRNA delivery system with the HA-tagged target transcript to detect cleavage efficiencies (Li et al. 2013, 2014a; Zhang et al. 2017).

MiRTrons for amiRNAs: MiRTrons are defined as miRNAs encoded by intronic sequences of protein coding genes (Meng and Shao 2012; Joshi et al. 2012; Fonseca et al. 2019). amiRNA-technology has been used to understand the process and accuracy of miRTron biogenesis and their ability to correctly target the candidate mRNA. Additionally, a combination of miRTrons and amiRNA technology has been tested as a potential tool for manipulation of multiple genes (Shapulatov et al. 2018). This technique entails engineering either a natural miRNA such as miR319a or amiRNAs against *PHYB*, *LUC* as a miRtron within *LUC*. The biogenesis and silencing efficiencies have been tested and found to be satisfactory (Shapulatov et al. 2018).

6.5 Summary and Conclusions

The architecture of plant genomes is constantly shaped by dramatic and recurrent genome duplication events that have resulted in polyploidy and increased copy number of genes. Coupled with extensive segmental and local duplications, large-scale expansion of genes is associated with genesis of homeologs that either trace divergent evolutionary paths or remain conserved. Differential selection pressures operative on such redundant genes determine their evolutionary fate. Duplicated copies may diversify to acquire novel roles, referred to as neo-functionalization, or decay as pseudogenized copies. Such diversification forms the molecular basis of phenotypic diversity and adaptations. Dissection of the role of gene(s) is a prerequisite to undertake crop improvement. However, redundant gene copies present in diploid and polyploid pose major challenges in functional genomics. Routine use of gain-of-function, and loss-of function strategies using constitutive over-expression or T-DNA based mutagenesis, respectively, are not applicable for traits controlled by redundant genes.

Gene silencing-based methodologies, mediated by small RNAs, such as siRNA and miRNA, have revolutionized the landscape of functional genomics in plants as these constitute potent gene silencing tools. siRNA-based gene silencing methods suffer from lack of specificity and often silence unintended transcripts resulting in undesirable off-target silencing. A heterogenous population of siRNA originating from a single dsRNA template, a hallmark of siRNA biogenesis, may result in scenarios wherein the siRNAs may share sequence complementarities with unintended targets. By contrast, precursors of microRNA genes give rise to a homogenous population of mature miRNAs thus minimizing instances of off-target silencing. The miRNA biogenesis machinery recognizes structural features of the imperfect hairpin-loop, and not the sequence per se, for correct processing. This permits substitution of natural miRNA/miRNA* with suitable 21-nt sequences without distorting the structural properties of the backbone to achieve biogenesis of amiRNAs. Researchers have exploited such fundamental features of miRNA biogenesis, mode, and mechanism of action to develop amiRNA-technology for precise gene silencing. Based on a set of well-defined rules that regulate biogenesis, incorporation of miRNA into RISC

coupled with knowledge on parameters determining target pairing, several automated or semi-automated programs have been developed for prediction of amiRNAs. Most of the popular tools such as WMD3, P-SAMS, and AmiRNA Designer, commonly used for design of amiRNAs provide access to transcriptome databases from a range of plants. This is necessitated to identify candidate transcript sequence for designing purposes, and to prevent off-target silencing through sequence comparison. Selection of appropriate criteria allows designing amiRNAs for selective targeting of alleles, homeologs, splice variants, and also redundant silencing of multiple genes for dissection of function. Several different natural miRNA precursor sequences have been tested as backbone in which the native miRNA/miRNA* is replaced with amiRNA/amiRNA*. There is a constant endeavour to modify and improve the amiRNA technology for wider applications. Toward this, several innovative approaches including use of chimeric backbone, synthetic polycistronic backbone, protein-based screening methods for amiRNA efficiency, and miRTron encoded amiRNAs have been developed. In last decade, amiRNA technology has been widely adopted for functional genomics, and trait manipulation in plants.

Acknowledgements Research laboratory of AS has received financial support from DBT, Govt. of India, SERB-DST, Govt. of India, and TERI-SAS, Delhi. Research in the laboratory of SD has been supported by financial assistance from DBT, Govt. of India, UGC, Govt. of India, and University of Delhi, Delhi.

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Chapter 7
Suppressor to Survival: RNAi as a Molecular Weapon in Arms Race Between Virus and Host

Anurag Kumar Sahu, Neeti-Sanan Mishra, and Rajarshi Kumar Gaur

Abstract The sessile nature of the plant kingdom has developed various defense strategies in response to viral attack with different degrees of success. RNA silencing is an essential part of the cellular mechanism that controls transcription and plays an important role in defense against virus infection. Viruses evade the plant defense strategy, by encoding various "suppressor" molecules, which interact with the small RNA species or the components of host RNA silencing machinery. The present chapter aims to compile the plant defense strategies with the help of small RNA-omics and their mode of action involved during plant–pathogen interactions. Finally, we discuss the specific applications of the RNAi-based strategies and various suppression proteins used during the counterattack of plant viruses.

Keywords VIGS · PTGS · Viral suppressors · RNA silencing · Small RNAs · Antiviral strategy

7.1 Introduction

The breeders face many challenges during the development of virus resistant plants such as the availability of virus resistance genes, the discovery of dominant molecular markers linking with the pathogen resistance, and introgression of resistance into susceptible cultivars. The incidence of the disease depends on vector population, environmental conditions, and plant developmental stage (Legg and Thresh 2000). Thus, it's very challenging to develop the durable cultivars against plant viruses.

For a long period of time, DNA has been considered the main nucleic acid and RNA as an intermediate molecule linking DNA with proteins or as a regulatory

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G. Tang et al. (eds.), *RNA-Based Technologies for Functional Genomics in Plants*,
Concepts and Strategies in Plant Sciences, https://doi.org/10.1007/978-3-030-64994-4_7

molecule. The discovery of long and short non-coding RNAs, with specific regulatory roles, has completely changed the overview of RNA biology.

Over the last few years RNA-mediated gene silencing has become an intensively studied biological phenomenon which involves gene regulation at different levels including suppression of transcription, transcript degradation, or translation inhibition (Agrawal et al. 2003; Mishra and Mukherjee 2007). The phenomenon has been found in natural virus resistance as well as in basic biological processes such as development, gene regulation, and chromatin condensation. The viruses are both initiators and targets of gene silencing (Pruss et al. 1997; Ratcliff et al. 1999). Virus Induced Gene Silencing (VIGS) has now been developed for the study of functional genomics to knock-down expression of endogenous plant genes by use of recombinant viruses (D. C. Baulcombe 1999; Kumar 2019).

RNA silencing is a highly sequence-specific gene regulation system, which plays an important role in maintaining the genome integrity in a wide variety of organisms. Similar to animal system, the plants also exhibit two main types of sRNAs, i.e., miRNAs and siRNAs (Bortolamiol et al. 2008; Vaucheret 2006). The production of sRNAs is either by double-stranded RNA (dsRNA) or from the folded structures by Dicer-like proteins (DCLs), and is guided by Argonaute (AGO) proteins (Ruiz-Ferrer and Voinnet 2009).

Post transcriptional gene-silencing (PTGS) is induced in response to virus attacks or by similar other factors such as viroids, satellite RNAs, defecting RNAs, and defecting-interfering RNAs (Yang et al. 2011). Plants utilize PTGS as an antibody-like mechanism to recognize and eliminate those molecular pathogens efficiently and specifically. Notably, every type of plant viruses (DNA, RNA-single or double stranded, of positive or negative genome polarity) has to pass through the RNA stage by forming a dsRNA, a progenitor of PTGS (D. Baulcombe 2004).

The activation of RNA silencing depends on RNA and DNA plant viruses via the formation of dsRNA and siRNAs (Ding and Voinnet 2007). Virus-induced RNA silencing occurs in three steps: initiation, amplification, and spreading (Voinnet 2008). When dsRNA is recognized by the same set of DCLs, responsible for the biogenesis of 21–24 nt endogenous siRNAs, silencing occurs (Xie et al. 2004). RNA-dependent RNA polymerases (RDRs) use single-stranded RNA (ssRNA) to synthesize long, perfect dsRNAs (Curaba and Chen 2008; Schiebel et al. 1998), which serve as a substrate for the formation of secondary siRNAs through DCLs (Voinnet 2008). This secondary pool of siRNAs initiates the systemic silencing that spreads throughout the plant (Molnar et al. 2010; Dunoyer et al. 2010). siRNAs associate with distinct AGO-containing effector complexes where they target DNA or RNA on complementarity basis (Hutvagner and Simard 2008).

To counteract viral RNA silencing, most plant and animal viruses have evolved silencing suppressor proteins (Silhavy and Burgyán 2004; Voinnet 2005). The molecular basis for suppressor activity has been identified for several viruses, including p21 of closteroviruses, HC-Pro of potyviruses, p19 of tombusviruses, and B2 protein of Flock House virus (FHV). All three silencing suppressors are dsRNA-binding proteins that interact physically with siRNA duplexes *in vivo* as well as *in vitro*,

p19, HC-Pro, and p21 uniformly inhibit RNA silencing initiator complex formation (Ghildiyal and Zamore 2009; Nayak et al. 2013; tenOever 2016).

The viruses evolved successful defensive strategy, where they acquired "suppressor" functions and restrict the host silencing machinery and systemic spread in the host. There is a wide diversity in the RNA Silencing Suppressor (RSSs) identified within the diverse viruses. In this chapter, we describe the different methods of screening the RSSs and focus on case studies of well characterized suppressors to follow their mechanism of action. The application of RSSs as important biological tools in research processes is also discussed. Finally, the chapter presents overall pictures about possible functions of siRNAs derived from endogenous viral elements (EVEs) and role of small RNA pathways during viral infection in plants.

7.2 Plant Antiviral Defense Mechanism

RNA silencing or RNA interference (RNAi) is a conserved antiviral defense system against pathogens including viruses. After infection, the first natural response is to recognize "foreign" molecules, followed by the onset of various signals to alert plant defense system (Ratcliff et al. 1997). A lot of information has emerged regarding the mechanism and machineries of RNA silencing and has been explained as a new tool for developing antiviral products in the area of agriculture and pharmacy (Haan et al. 1992; Vlugt et al. 1992). Wide-range of studies have been performed in the agriculture sector to improve the quality of foods, yields, and to remove undesirable metabolites (Davies 2007). The use of RNAi showed an effective resistance against plant-parasitic nematodes (Sindhu et al. 2008), coleopteran, and lepidopteran insects (Gu and Knipple 2013). In the medical sector, efficient *in-vivo* delivery of siRNAs for therapeutic benefit has been demonstrated in mice or bovine models (Uprichard 2005).

The first RNA silencing concept came in existence when Napoli and his co-workers were trying to increase the flower pigmentation of petunia plants to over-express the chalcone synthase (*chs*) gene (Napoli et al. 1990; Van der Krol et al. 1990). After that, RNA silencing was used for pathogen-derived resistance (PDR) to produce virus resistant plants where recovered parts correlated with reduction of viral mRNA in the cytoplasm. This indicated that the expression of viral proteins was not only required for virus resistance, but also for untranslatable viral RNA which plays an important role in resistance during pathogen attack (Lindbo and Dougherty 1992; Haan et al. 1992; Vlugt et al. 1992).

To explain the mechanism of silencing, Hamilton and Baulcombe (1999) proved that plants with a silenced transgene accumulated small dsRNA molecules identical to the transgene. They observed that the silencing of *uidA* gene (encoding for β-glucuronidase, GUS) could prevent the accumulation of *Potato Virus* X (PVX), suggesting a role in antiviral defense mechanism. The basic mechanism of RNA silencing pathway is the cleavage of dsRNA structure into 21–24 nt length

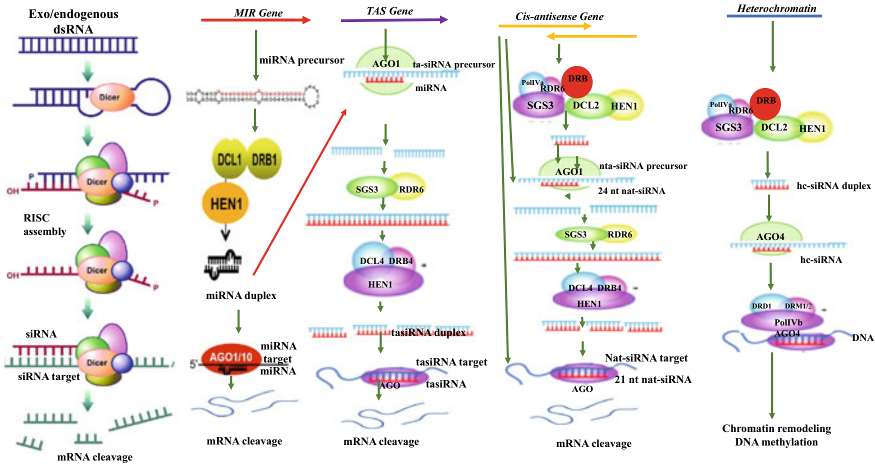

Fig. 7.1 miRNA and various siRNA biogenesis pathways in plants. The arrow connecting the miRNA and ta-siRNA pathway indicates the miRNA triggers the ta-siRNA pathway. Multiple arrows in the nat-siRNA pathway indicate the RNA transcribed from the sense strand is being used at multiple places in the pathway. mRNA cleavage in different silencing pathways is shown and the DNA modification steps in the hc-siRNA pathway involve TGS (Modified from Vaucheret 2006)

small RNAs by DCLs (type III RNA endonucleases), RNA-dependent RNA polymerase (RdRP), and AGO. The small RNAs includes the siRNAs (small interfering RNAs) with their various sub-types, viz., ta-siRNA (trans acting siRNAs), ra-siRNA (repeat associated siRNAs), vi-siRNA (viral siRNAs), nat-siRNA (natural antisense siRNAs), etc., and the miRNAs (microRNAs) (Fig. 7.1). The RNA silencing occurs either at cytoplasmic level known as PTGS or at nuclear level called Transcriptional Gene Silencing (TGS).

7.2.1 PTGS as Antiviral Defense

RNA silencing is an innate antiviral defense system triggered in response to viral infection. The dsRNAs derived from virus replication act as pathogen-associated molecular patterns (PAMPs) and activate the pattern-recognition receptors (PRRs) and DCL enzymes to make siRNAs (Zvereva and Pooggin 2012). The accumulated siRNAs during viral infections are either loaded into the RISC effector complex to guide specific localized silencing (Hammond et al. 2000, 2001; Elbashir et al. 2001; Nykänen et al. 2001; Tang et al. 2003; Vazquez et al. 2004) or lead to systemic silencing through the formation of transitive siRNAs (Agrawal et al. 2003).

7.2.2 TGS as Antiviral Defense

The silencing pathway also operates at the nuclear level and is directed by the 21–24 nt siRNAs and miRNAs (Xie and Yu 2015; Teotia et al. 2017). It involves silencing of heterochromatin by DNA methylation and modification of histone proteins at post-transcriptional level (e.g. H3- methylation at lysine 9). This DNA methylation confers gene silencing and plays crucial roles in plant development and defense against viruses generated by DCL3 with the help of AGO4 and RdRP2 (Agrawal et al. 2003; Mishra and Mukherjee 2007) and transgenes by RNA-directed DNA methylation (RdDM). Apart from antivirus defense, RdDM mechanism guides chromatin modifications and silences transposons in plants (Fig. 7.1).

7.3 Small RNA Pathways in Antiviral Defense

RNA silencing is an inducible defense pathway that uses siRNAs for specific targets resulting in the inactivation of foreign nucleic acids invading the cells. The defensive role of siRNAs against plant viruses became clear nearly a decade ago through two experimental evidence: first, during virus infection long dsRNAs are generated as replicative intermediates or convergent transcription of viral RNAs that serve as a potent trigger molecules for RNA silencing, suggesting the existence of an RNA-directed antiviral defense mechanism (Lindbo et al. 1993). Second, plant viruses were found to encode potent suppressors of silencing, an effective counter-defense strategy (Anandalakshmi et al. 1998; Brigneti et al. 1998; Kasschau and Carrington 1998).

7.3.1 Virus Induced Gene Silencing

The siRNA produced during the first infection serves as "molecular memory" to protect the plants against the similar or related virus threats (Pruss et al. 1997; F. G. Ratcliff et al. 1997, 1999). This cross protection phenomenon now named as VIGS was first reported by plant virologists during 1920. Soon after that VIGS was used as an effective defensive tool during the plant virus infection (Ratcliff et al. 1997). It was believed that plant viruses replicate via dsRNA intermediate, the principal inducer of siRNA system. In the cross protection, it was observed that degradation of desired mRNA is due to over expression of certain genes while using viral vectors. Thus, the ability of recombinant viruses to knock down expression of endogenous genes was known (Ruiz et al. 1998; Baulcombe 1999). VIGS is known to be an efficient tool for the study of functional genomics in comparison to agroinfiltration and biolistic gene gun methods. It is a cost-effective experiment generating rapid characterization of phenotypes with an easy transformation. Several plant viruses

Table 7.1 List of viruses used for the construction of VIGS vectors

Virus backbone	References
DNA virus	
Tomato Golden Mosaic Virus (TGMV)	Peele et al. (2001)
Cabbage Leaf Curl Virus (CaLCV)	Turnage et al. (2002)
Tomato Leaf Curl Virus (ToLCV)	Pandey et al. (2009), Huang et al. (2009)
Tomato Leaf Curl Virus satellite	Li et al. (2004)
Satellite DNAβ of Tomato Yellow Leaf Curl China Virus	Tao and Zhou (2004)
Tobacco Curly Shoot Virus	Li et al. (2018)
RNA virus and their satellites	
Tobacco Mosaic Virus (TMV)	Kumagai et al. (1995), Lacomme et al. (2003)
Satellite Tobacco Mosaic Virus (STMV)	Gosselé et al. (2002)
Potato Virus X (PVX)	Ruiz et al. (1998)
Tobacco Rattle Virus (TRV)	Ratcliff et al. (2001)
Barley Stripe Mosaic Virus (BSMV)W	Holzberg et al. (2002)
Pea Early Browning Virus (PEBV)	Constantin et al. (2004)
Brome Mosaic Virus (BMV)	Ding et al. (2006)
Bean Pod Mottle Virus	Zhang et al. (2006)
Cucumber Mosaic Virus (CMV)	Sudarshana et al. (2006)
Tomato Mosaic Virus (ToMV)	Andolfo et al. (2014)

including *Tobacco mosaic virus* (TMV) have been modified for the study of VIGS. TMV was the first VIGS-based vector used for *phytoene desaturase (pds)* silencing in *Nicotiana benthamiana* (Kumagai et al. 1995). A list of commonly used VIGS vectors is provided in Table 7.1.

7.3.2 MicroRNA as an Antiviral Defense

miRNAs have emerged as important players regulating the expression of both host and viral genes at post-transcriptional levels (Fig. 7.1) (Naqvi et al. 2010, 2011; Pradhan et al. 2015). These non-coding RNAs (20–24 nt) bind to and initiate the degradation of complementary mRNAs followed by translational inhibition. A specific set of miRNAs are differentially expressed during virus infection in different

plant-virus interactions (Khraiwesh et al. 2012; Pradhan et al. 2015). Expression of several miRNAs such as miR159, miR156, miR164, miR166, miR160/167, and miR170 was found to modify the targeted transcription factors related to development like *LEAFY*, *SQUAMOSA PROMOTER BINDING LIKE PROTEIN*, Homeodomain-leucine zipper, *AUXIN RESPONSIVE FACTOR*, SCARECROW among others. Although various studies have been done on various plant species unraveling the significance of miRNAs during virus infection, the precise function of the most is still unidentified.

7.3.3 Suppressor of RNAi

Plant viruses infect wide variety of plant species resulting in a range of symptoms and damages by invading the plant defense system. They encode protein molecules known as "suppressors" and interfere at different levels of silencing pathways (Voinnet et al. 1999; Shi et al. 2002). This interaction results in infection and replication of plant viruses within the host cell and its systemic spreading. Diverse group of viral suppressors (RSSs) including coat protein, movement protein, and proteases have been identified with no sequence homology (Anandalakshmi et al. 1998; Brigneti et al. 1998; Kasschau and Carrington 1998). It has also been identified that the activity of suppressors is coupled with the host transcription factors for effective results (Hartitz et al. 1999). The RSSs target the various effectors of the silencing pathway, such as viral RNA recognition, dicing, RISC assembly, RNA targeting, and amplification (Fig. 7.2 and Table 7.2). Management of these multifunctional RSSs can be used to eradicate the virus threat.

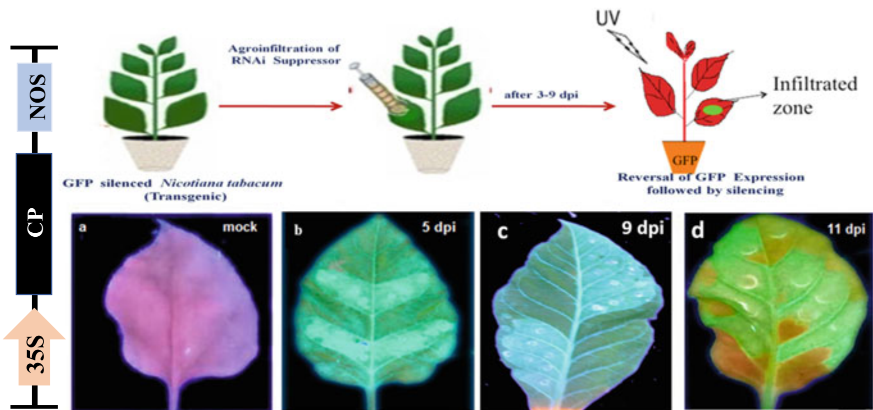

Fig. 7.2 Schematic representation of GFP reversal assay—Agrobacterium transient assay to show that ChilCV-AC2 acts as a suppressor of RNA silencing. Panel 1 shows the non-infiltrated leaf of GFP silenced tobacco leaf. Panels 2, 3 and 4 show different time point, GFP silenced leaves of tobacco agroinfiltrated with pCAMBIA1302- ChilCV-AC2. All the pictures were taken after 5, 9 and 11 dpi under UV light

Table 7.2 List of RNA silencing suppressors identified from different viruses

Virus genus	Virus	Suppressor	Other viral function	References
DNA viruses				
Begomovirus	African cassava mosaic virus	AC2	Transcriptional activator	Voinnet et al. (1999)
	Tomato yellow leaf curl virus	C2	Transcriptional activator	Vanitharani et al. (2004)
Curtovirus	ACMV TGMV TYLCV-C BCTV	AC2 AC4 AL2 C2	Transcriptional activator	Wang et al. (2003) Dong et al. (2003) van Wezel et al. (2002)
Positive strand RNA viruses				
Carmovirus	Turnip crinkle virus	P38	Coat protein	Thomas et al. (2003)
Potyvirus	Potato virus Y Tobacco etch virus Turnip mosaic virus	HC-Pro HC-Pro HC-Pro	Long-distance movement Polyprotein processing	Voinnet et al. (1999) Lu et al. (2004) Lu et al. (2004)
Cucumovirus	Cucumber mosaic virus Tomato aspermy virus	2b 2b	Movement	Lu et al. (2004)
Sobemovirus	Rice yellow mosaic virus	P1	Movement	Brigneti et al. (1998) Lucy et al. (2000)
Tombusvirus	Artichoke mottled crinkle virus	P19	Pathogenesis determinant	Yelina et al. (2002) Pfeffer et al. (2002)
	Carnation Italian ringspot virus	P19	Movement	Voinnet et al. (2000)
	Tomato bushy stunt virus	P19		Brigneti et al. (1998)
	Cymbidium ringspot virus	P19		Anandalakshmi et al. (1998)
Tobamovirus	Tomato mosaic virus	P130	Replication protein	Dunoyer et al. (2004)
Closterovirus	Beet yellows virus	P21	Replicational enhancer	Qu et al. (2003)
Pecluvirus	Peanut clump virus	P15	Movement	Voinnet et al. (1999)
Poleovirus	Beet Western yellows virus	P0	Pathogenesis related	Reed et al. (2003)
Potexvirus	Potato virus X	P25	Movement	Voinnet et al. (1999)
Negative-strand RNA viruses				
Tospovirus	Tomato spotted wilt virus	NSs	Virulence	Takeda et al. (2002) Bucher et al. (2003)
Tenuivirus	Rice hojablanca virus	NS3	None known	Bucher et al. (2003)

7.4 Approaches for the Identification of RSS

Simple and reliable functional assays to detect RSSs activity greatly accelerated their discovery. Functional identification and characterization of RSS facilitated to understand its involvement in the RNA silencing pathway. It also provided insights into the evolutionary arms race between the host and the pathogens during pathogenicity. For identification and characterization of RSSs, three major screening processes are described based on the role of viral proteins in RNA-mediated silencing of a reporter gene: (1) a gene, (2) the inducer, and (3) putative suppressor viral proteins. Delivery of the RSS can be done either by agroinfiltration or by crossing the silenced plant with the one expressing RSS or by using viral vectors (Cao et al. 2005; Niu et al. 2009). Some of the commonly used assays for RSS identification are discussed below:

7.4.1 Agroinfiltration and Reversal Assays

Agrobacterium-based transient expression tool is a very convenient and efficient method for generating silenced plants. This method is regularly used for initial verification of suppressing activity of viral proteins by using reporter gene like beta-glucoronidase (GUS) and green fluorescent protein (GFP). The infiltration of RNAi constructs against the reporter gene will initiate RNA silencing and typically it becomes silenced after three to five days. In the presence of a candidate RSS protein, there will be suppression of silencing and the reporter gene expression will remain at its high level or even for increased time duration.

7.4.2 Reversal of Transgene Induced Silencing

The screening of viral RSS proteins was achieved based on the rescue of RNA-mediated silencing of reporter genes like GFP or GUS. The most commonly used *in planta* assay is based on transgenic tobacco plants constitutively silenced for a reporter gene (Elmayan and Vaucheret 1996). The RSS activity has been found to vary for the homologous proteins encoded by virus of the same genus (Mangwende et al. 2009; Sundaresan et al. 2020). The RSS activity can be assayed by restoration of reporter gene expression, indicating that the tested construct encodes a RSS (Fig. 7.2). This also enables to know the specificity of siRNA-RSS interaction and the role of RSS based on site-directed mutagenesis.

7.4.3 *Crossing Assay*

Another method involves generating stable transgenics of the silenced reporter gene and overexpressing the candidate viral RSS (Anandalakshmi et al. 1998; Kasschau and Carrington 1998; Kasschau et al. 2003; Chapman et al. 2004; Dunoyer et al. 2004). It involves a cross between those two transgenics and the resulting progeny is screened for the reporter gene expression (Anandalakshmi et al. 1998; Kasschau and Carrington 1998). However, this is an intensive laborious method and the overexpression of RSS in the plant affects seed formation and developmental defects (Anandalakshmi et al. 1998).

7.4.4 *Grafting Assay*

Grafting assay is found to be most reliable to study suppression of silencing in plants but time consuming. The systemic movement of RSS can be assayed by grafting of a reporter gene-expressing scion to a silenced rootstock. Due to the spread of RSS activity there will be no effect on the reporter gene expression in the scions.

7.5 Mechanism of RNA Silencing Suppression

In the PTGS, the complex machinery recognizes the viral RNA, resulting in its degradation. RSS initially acts on functional components of the mechanism and suppresses the siRNA activity, thus breaking plant defense response (Table 7.2 and Fig. 7.2) (Carrington et al. 2001; Li and Ding 2001; Baulcombe 2002; Roth and Breaker 2004; Silhavy and Burgyán 2004; Karjee et al. 2010). Despite the fact and knowledge of RSS, the fundamental mechanism of this virus–host interaction is still a mystery. The common mechanisms of these interactions are discussed below:

7.5.1 *Binding of Long dsRNAs: Inhibition of the Dicing Steps*

Long and small dsRNA serve as a major inducer and effector molecule, respectively, and inhibit the host defense system. The binding of RSS either protect it from the DCL-dependent degradation (long dsRNA) or prevents their sorting into the AGO containing RISC complex (small dsRNA) thus inhibiting the processing of sIRNAs (Mérai et al. 2006). Interaction of these dsRNA was much explored in diverse groups of viruses like tombusvirus P19, closterovirus P21, carmovirus CP, pecluvirus p15, hordeivirus QB, potyvirus HC-Pro, CMV-2b (cucumber mosaic virus) (Goto et al. 2007; Lakatos et al. 2006; Singh et al. 2009; Ye and Patel 2005). The mechanism of

RNA binding for these suppressor proteins varies from the type of RSS (Chen et al. 2008). P19 protein forms a caliper-like structure by extending its S-sheet surface and R-helix whereas TAV2b interacts with siRNA by forming a pair of hook-like structures. Unlike P19 and TAV2b, few RSSs (e.g., p14, FHV-B2) interfere with the function of miRNAs and siRNAs (Singh et al. 2009).

7.5.2 Binding to Biogenesis Components

Several RSSs have been found to directly affect the small RNA biogenesis by interacting with DCLs. P38 of *Turnip Crinkle Virus* and P6 of CMV suppress the activity of host DCL4 enzyme and inhibit defense response activities (Deleris et al. 2006; Haas et al. 2008). During the virus threat, host RDRs (RDR1 and RDR6) have been found to be involved in the siRNA biogenesis. These secondary siRNAs provide essential antiviral immunity against CMV (Garcia-Ruiz et al. 2010; Ruiz-Ferrer and Voinnet 2009; Wang et al. 2010) and MYMIV-AC2 (Mungbean yellow mosaic India virus) (Kumar et al. 2015).

7.5.3 Viral Suppressors Preventing RISC Assembly

RSS interacts directly and indirectly to the AGO protein of RISC, thus preventing the assembly and suppression activity of RISC. The 2b protein of CMV and Polerovirus-encoded P0 suppressor destabilizes AGO1 by interacting with its PAZ domain and partially with PIWI domain, inhibiting the slicing activity of AGO1 (Bortolamiol et al. 2008; Ruiz-Ferrer and Voinnet 2009; Zhang et al. 2006). The X-ray crystallographic study of TAV-2b-siRNA showed that 2b binds to long dsRNA (Goto et al. 2007) and inhibits the production of viral secondary siRNAs (Diaz-Pendon et al. 2007). The RSSs are believed to follow different pathways while interacting with siRNAs and miRNAs or AGO proteins. In the presence of RSSs, the plants are not able to suppress the spread of the viral infection as siRNAs are inactivated before the incorporation into the RISC (Havelda et al. 2005) Whereas, in the absence of siRNA-binding-RSSs, siRNAs activate the RISC and move faster than viral proteins, thereby establishing the antiviral immunity and faster recovery of the plant (Havelda et al. 2003, 2005).

7.5.4 Interference with DNA Methylation

Some of the RSSs have the ability to cause reversal of TGS. For e.g., 2b protein of CMV (Shan-Dong isolate) suppresses RNA-dependent DNA methylation (RdDM) by interacting with siRNAs involved in the process of AGO proteins in nucleolus (Duan et al. 2012). In *Arabidopsis,* out of ten identified AGO proteins, only four are

very well characterized and explored. AGO1 and AGO7 possess the slicer activity of siRNA-guided endonucleolytic cleavage whereas AGO4 and AGO6 are required for the establishment of DNA methylation during TGS (Baumberger and Baulcombe 2005; Montgomery et al. 2008; Zheng et al. 2007; Zilberman et al. 2004). Another best example for reversal of TGS is by AC2 protein of Begomovirus and Curtovirus genera, in which AC2 protein inactivates adenosine kinase, reducing the production of methyl donor (SAM) and thus releasing TGS (Buchmann et al. 2009).

7.6 Components of RNAi Silencing Machinery

Based on small RNA omics, transcriptomics, and phylogenomics, the endogenous small regulatory RNAs have been divided into miRNAs and siRNAs. These miRNAs and siRNAs are generated by ssRNA or dsRNA with the help of DCL and AGO proteins (Fang and Qi 2016; Rogers and Chen 2013). Plant endogenous siRNAs are further divided into hairpin-derived (hpsiRNAs), natural antisense transcript (natsiRNAs), phased (phasiRNAs), and trans-acting siRNAs (tasiRNAs). These RNA transcripts are cleaved as directed by miRNA, recruiting the RDR6 or RDR1 of RDR family for generating dsRNA. These processed dsRNAs, further with the help of DCL4 and/or DCL 2, produce 21–22 nt siRNAs (Fig. 7.1).

7.6.1 Cis-*Acting siRNAs as a Silencing Tool*

RNA-based immune system showed that the endogenous or exogenous nucleic acids are controlled by *cis*-acting 24 nt siRNAs that are generated from DCL3 from these endogenous and exogenous nucleic acid molecules and degrades RNA from which they are derived. This is referred to as auto-silencing by *cis*-acting siRNAs (Dunoyer and Voinnet 2005; Gustafson et al. 2005; Lu et al. 2005; Worrall et al. 2019) (Fig. 7.1).

7.6.2 Trans-*Acting siRNAs as a Silencing Tool*

Trans-acting siRNAs or ta-siRNAs are derived from TAS loci processed from DCL4. AGO- mediated miRNA direct the cleavage of the non-coding transcripts to produce 21nt tasiRNAs with the help of RDR6 followed by DCL4. Based on the binding site on miRNAs, the TAS gene family is classified into four, where TAS1, TAS2, TAS4 require one binding site while TAS3 needs two binding sites for generating tasiRNAs (Rhoades et al. 2002).

7.6.3 *Natural* cis-*Acting siRNAs as a Silencing Tool*

Cis-nat-siRNA or natural cis-antisense siRNAs are another class of silencing tool produced by the transcription of an antisense gene. There are two types of nat-siRNAs, i.e., 24 nt nat-siRNA and 21nt-nat-siRNA. 24 nt nat-siRNAs are produced during the environmental stress whereas 21 nt-nat-siRNAs are produced from the cleavage of 24 nt nat-siRNAs by DCL1 and its function is still not know (Borsani et al. 2005) (Fig. 7.1).

7.6.4 Anti-sense RNA

Antisense RNA is an RNA transcript that is complementary to endogenous mRNA and similar to negative-sense viral RNA. Anti-sense RNA (asRNA) sequences were used before the discovery of the engineering resistance against geminivirus (Day et al. 1991). There have been numerous models developed for PTGS involved with antisense and virus resistance. The expression of viral-derived antisense in transgenic plants appear to induce more resistance and triggers a form of PTGS.

7.6.5 Targeting RNA Components of Silencing

p19 binds to the siRNAs efficiently and thus used for the isolation of siRNAs. This dimeric protein has a nanomolar affinity with siRNAs and suppresses RNA interference. There are many suppressor proteins, for example, Potato Virus X (PVX) p25 which acts similar to p19 and targets the siRNAs, resulting in an increased infection in the plants (Silhavy et al. 2002; Voinnet et al. 2000).

7.6.6 Targeting Protein Components of Silencing

Sometimes suppressors proteins also target the host protein thus inhibiting the silencing system. For example, HC-pro interacts with calmodulin related protein (rgs-CaM) of the host and suppresses the RNA interference. rgs-CaM is a regulator of gene silencing and acts as an antiviral and binds to the HC-Pro and CMV 2b and degrades the viral RSS activity (Jeon et al. 2017). Besides the activation of immune system in plants via rgs-CaM, inhibition of plant growth was also observed by its overexpression. This may be the cause of evolution of receptor- based immune receptors against the pathogens, which get induced only when required.

7.6.7 *Modifying Expression of Host Genes*

Viral suppressor protein alters the activity of host proteins for the establishment of diseases (Dong et al. 2003; Voinnet et al. 1999). For example, AC2/TrAP interacts with host SNF1 and adenosine kinase, which is believed to be active during the defense response (Hao et al. 2003). Geminivirus uses the host histones for their DNA packing forming a chromosome-like structure known as minichromosome. Castillo-Gonzalez et al. (2015) demonstrated that KRYPTONITE enzyme (SET domain family) binds to these minichromosomes and methylates virus histones, thus stops virus replication. In the presence of TrAP, the activity of KRYPTONITE is blocked thus allowing the replication of viruses.

7.7 Implications for RNA Silencing Suppressor

Viruses regulate host cellular processes through the deployment of numerous RSSs (Bisaro 2006). Due to diversity and multilateral actions of RSSs, it requires an effective defense strategy for plants to evade virus counter-defense. RNAi-based silencing of RSSs is desirable to target the main determinant of pathogenicity in virus infection cycle. Effective negative regulation of RSSs deploying siRNAs (Praveen et al. 2010; Ramesh et al. 2007), artificial miRNAs (amiRNAs) (Q.-W. Niu et al. 2006), and artificial transacting siRNAs (A. Singh et al. 2015) have imparted some virus resistance. However, widespread infections by geminivirus under field conditions combined with other infections might synergize or complement the effect of unrelated viruses (Vanitharani et al. 2004). There are following ways where RSSs can be used as.

7.7.1 *RSSs as Tools Unraveling the Molecular Basis of Silencing*

Due to the versatile range of actions targeting every aspect of RNA silencing, RSSs can be used to unravel mechanistic understanding of both endogenous and antiviral RNA silencing pathways. DCLs work in a hierarchical fashion and there is a complex autoregulation of silencing pathways through feedback loops governed by DCLs and AGOs activities (Molnar et al. 2010). This TCV-p38-based method could prevent siRNA but not miRNA loading, suggesting at the localization of AGO1 in distinct cellular pools (Dunoyer et al. 2010). HC-Pro usage showed the presence of endogenous regulators of silencing like rgs=CAM (Anandalakshmi et al. 2000). Furthermore, the study of RSSs has helped to understand the complex relationship between RNA, protein-based immunity, and hormone regulation during pathogenesis.

7.7.2 RSSs as Molecular Probes

In plants, local silencing is also spread over longer distances involving two distinct siRNAs species (21 nt and 24 nt). It has been shown that the two siRNA species are regulated by some silencing suppressors at different levels (A. Hamilton et al. 2002). A surface plasmon resonance (SPR)-based miRNA sensing method was developed which used the silencing suppressor, p19. In that method, the RNA probes are immobilized on gold and miRNA:probes duplexes are recognized by p19. Similarly, p19 was used to detect miRNA in blood serum through protein-facilitated affinity capillary electrophoresis (Berezovski and Khan 2013). These highly sensitive methods have an edge over PCR-based techniques as they rule out amplification bias and utilize viral suppressors for RNA silencing.

7.7.3 Enhancing Transgene Expression

RNA silencing greatly influences transgene expression and also interferes with the production of transgenic commercial crops with predicted yield phenotypes. It is also deployed through the miRNA pathway where long RNAs with strong secondary structures are processed by DCL1 into 21–24 nt small RNAs (Reinhart and Bartel 2002). Because of the similarity between the miRNA and siRNA pathways, it was speculated that RSSs could cause developmental defects in plants. Indeed, HC-Pro of *Turnip Mosaic Virus* (TuMV) in Arabidopsis was shown to reduce the accumulation of miRNAs with concomitant increase in the expression of target genes, resulting in morphological defects similar to those of *dcl-1* partial mutants (Kasschau et al. 2003; Mallory et al. 2001; Pruss et al. 1997). This approach is fast, flexible, and reproducible and is suitable for *in planta* production of heterologous proteins. However, this approach has a limitation as heterologous protein expression usually gets reduced after 2–3 days due to activation of RNA silencing machinery. Hence, co-delivery of silencing suppressors in *Agrobacterium* cultures was found to enhance ectopic gene expression (Johansen and Carrington 2001; Voinnet et al. 2000). Because of this, p19 is widely used in the production of heterologous proteins in plants for industrial and research purposes.

7.7.4 Development of Antiviral Strategies

RSSs are major defense factors against the antiviral RNA silencing strategies. Virus mutants with attenuated ability to produce RSSs showed only mild disease symptoms. The chemical compound impeding the suppression activity may act as an important virucide against viruses infecting crops of importance (Shimura et al. 2008). Artificial microRNA (amiRNA) technology is based on designing miRNAs artificially against

any gene of interest by mimicking the secondary structure of endogenous miRNA precursors (Ossowski et al. 2008; Sablok et al. 2011). The antiviral remedy involves generating plants producing amiRNAs against pre-coat and coat protein transcripts of ToLCNDV in tomato (Van Vu et al. 2013). The amiRNA technology is being used to target viral-encoded RSS transcripts (Tiwari et al. 2014). Apart from amiRNAs, artificial transacting siRNAs have been considered effective in downregulating RSSs (A. Singh et al. 2015). However, these RNAi-based approaches may not circumvent infection in case the plant is infected by multiple viruses.

One recent approach to enhance plant immunity against infecting geminiviruses involves genome editing using clustered regularly interspaced short palindromic repeats (CRISPRs)/CRISPR associated 9 (Cas9) protein (Baltes et al. 2015; X. Ji et al. 2015; Y. Ji et al. 2007). The approach enables targeted modification of viral genomic DNA with the transgenic expression of a single guide RNA and Cas9 endonuclease. This approach has been activated against beet curly top virus (BCTV), Merremia mosaic virus (MeMV), and tomato yellow leaf curl virus (TYLCV) (Sharma and Prasad 2017).

7.7.5 Molecular Farming

Molecular farming in plants has several advantages over the traditional production systems (Chu and Robinson 2001). RSSs can be employed efficiently to limit transgene silencing and help produce higher levels of diverse products like vaccines and pharmaceuticals, high-nutritive foods, high-value products among others (Naim et al. 2012). Plants are being used as biofactories for production of biochemical compounds (Fischer and Emans 2000). The first protein made in plants was human growth hormone in transgenic tobacco in 1986 (Barta et al. 1986). To minimize expression variation due to position effect, gene-copy number and other factors, co-transformation of plants with a gene of interest and a viral-silencing suppressor such as p19, enhanced protein expression up to 50-folds or more (Voinnet et al. 2003).

7.8 Conclusions

RNA-dependent silencing of target genes serves as a key regulatory mechanism to control gene expression to help plants adapt better to stresses and give higher yield. The most recent data indicate that RSSs regulate the multiple layers of the complex defense, counter-defense, and counter-counter-defense between host and pathogen. Apparently it is clearer now that RSSs are not just simply blockers of RNA silencing but interconnect antiviral silencing, protein-based immunity, hormone signaling, RNA metabolism, and subcellular organization (Pumplin and Voinnet 2013). Beyond model plants like Arabidopsis and tobacco, such technologies can significantly benefit crop breeders.

Due to the ability of cross-kingdom infection of certain viruses, an analysis of RSSs in their natural virus backgrounds is essential. Further characterization of the key domains in these viral proteins will help develop novel antiviral tools.

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Chapter 8
An Improved Virus-Induced Gene Silencing (VIGS) System in Zoysiagrass

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Abstract Virus-induced gene silencing (VIGS) is a powerful RNA-silencing technique for transient gene manipulation and functional verification. However, the procedure of its application among dicot and monocot species varies based on types of vectors, plant susceptibility, as well as inoculation methods. Here, we described a simple yet efficient tobacco rattle virus (TRV)-based VIGS system for functional analysis of genes in zoysiagrass (*Zoysia japonica* cv. "Zenith"), an important warm-season turfgrass species, using *Nicotiana benthamiana* as an intermediate host. VIGS of the *PDS* reporter gene resulted in a characteristic photo-bleaching phenotype in majority of the inoculated leaves up to 70% reduction of expression of the endogenous *phytoene desaturase* (*PDS)* gene in zoysiagrass. Our protocol provides a fast and efficient toolbox for high-throughput functional genomics in zoysiagrass species, which could potentially be applied to other warm-season turfgrass species.

Keywords Virus-induced gene silencing · Tobacco rattle virus · Zoysiagrass · *Agrobacterium tumefaciens* · Functional genomics · Phytoene desaturase · Post-transcriptional gene silencing

8.1 Introduction

Since its emergence, virus-induced gene silencing (VIGS) has been providing a powerful approach for plant genetic and functional characterization, in a timely manner. VIGS is basically taking advantage of post-transcriptional gene silencing (PTGS), which in plants is dependent upon a relatively high degree of nucleotide homology between RNA transcript and target gene sequence (Ding 2000; van den Boogaart et al. 1998). In brief, VIGS mechanism is co-opted to target host mRNAs by including a fragment of target gene into a modified viral genome. The viral replication of chimeric double-stranded intermediates, including the target gene fragment, are produced and then recognized by RNA-induced silencing complex (RISC) as

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G. Tang et al. (eds.), *RNA-Based Technologies for Functional Genomics in Plants*, Concepts and Strategies in Plant Sciences, https://doi.org/10.1007/978-3-030-64994-4_8

foreign invaders in plant. Subsequently, the Dicer-like proteins process them into small interfering RNAs (siRNAs), which serve as specific templates to target any transcripts with highly similar sequences for degradation (Baulcombe 1999). Therefore, it is designed to manipulate target gene function utilizing the power of plant–virus interaction, without genome modification in plant itself.

The VIGS system has been successfully established in tobacco by suppressing phytoene desaturase (PDS) gene (Kumagai et al. 1995). Many plant DNA and RNA viruses have been modified as VIGS vectors and used to investigate gene functions. In eudicot species, VIGS has been reported to be adapted to tomato, Arabidopsis, petunia, potato, cassava, tobacco, soybean, vine, cotton, rose, apricot, almond, and sweet cherry, etc. (Burch-Smith et al. 2006; Chen et al. 2004; Faivre-Rampant et al. 2004; Fofana et al. 2004; Liu et al. 2002a, 2013; Kawai et al. 2016; Muruganantham et al. 2009; Nagamatsu et al. 2007; Qu et al. 2012; Tang et al. 2010; Tian et al. 2014; Turnage et al. 2002; Zhang and Ghabrial 2006; Zhao et al. 2020a, b, c). The most widely used VIGS vector is based on tobacco rattle virus (TRV), due to its vigorous viral replication and wide host spectrum (Burch-Smith et al. 2006; Liu et al. 2002b). In comparison to the many choices of VIGS systems adapted for dicots, only a few VIGS systems have been established for monocot species. Four RNA viruses, barley stripe mosaic virus (BSMV), brome mosaic virus (BMV), bamboo mosaic virus, foxtail mosaic virus (FoMV), and one DNA virus, rice tungro bacilliform virus (RTBV), were modified for VIGS in several monocot species (Ding et al. 2006; Holzberg et al. 2002; Liou et al. 2014; Liu et al. 2016; Meng et al. 2009; Pacak et al. 2010; Purkayastha et al. 2010; Tai et al. 2005; Yuan et al. 2011). BMV-based vectors have been extensively used for VIGS in some monocot species, but are not suitable for others, or not for all cultivars within a single host species (Ding et al. 2006; Pacak et al. 2010). Considering the advantages of higher viral activity and wider host range using TRV-based VIGS system, it is not uncommon for researchers to incorporate VIGS vector that was designed for eudicot species when they need to develop a better VIGS system for monocot plants. Recently, efforts have been put to use TRV-based VIGS system in monocot species. For instance, Singh et al. (2013) first reported their success of using TRV-based VIGS on gladiolus, and provided optimized protocol. In another report, Zhang et al. (2017) reported a rapid and whole-plant level gene silencing phenotype in both wheat and maize, using the TRV-based VIGS system. Therefore, TRV-based VIGS system has been proven to be fast, convenient, and efficient in such two monocot plants, which will also inspire future studies on other monocot species.

Zoysiagrass (*Zoysia* spp.) is one of the important warm-season turfgrass species. Due to its superior morphological characteristics and turf performance, zoysiagrass has been extensively used in home lawns, golf courses, sports fields, recreational parks, and other land surfaces (Patton et al. 2004). Besides its commercial use, zoysiagrass is also an ideal plant material to explore growth regulation and responses to environmental stresses in perennial monocot species (Huang et al. 2014). Efforts have been taken for studying gene functions by using polyethylene glycol (PEG)-mediated

direct gene transfer into protoplast (Inokuma et al. 1998), or by following the traditional plant tissue culture and Agrobacterium-mediated transformation in zoysiagrass. Toyama et al. (2003), found that zoysiagrass tissue culture could produce up to four types of calluses. Among them, only type 3 (yellow, compact, and friable) callus was suitable for Agrobacterium-mediated transformation. The selection criteria for such callus type requires extensive experience, and may also be subjective. A newer version of Agrobacterium-based transformation was recently reported in zoysiagrass, using stolon nodes as transforming materials (Ge et al. 2006). The callus formation process was bypassed, and the transformation period was shortened from 5 months to 3 months. However, the transformation frequency in this system was only up to 6.8%, which is not suitable for high-throughput functional genomic study. On the other side, VIGS assays using DNA viruses have also been exploited. For example, a rice tungro bacilliform virus (RTBV)-based VIGS system could lead to an average of 30.5–42.4% reduction of the *PDS* gene expression in half of the inoculated zoysiagrass plants (Zhang et al. 2016).

In this chapter, we described a protocol to use the leaf sap from TRV-infected *N. benthamiana* leaves to inoculate zoysiagrass. This improved VIGS method showed that the TRV-based system is able to suppress *PDS* reporter gene expression up to 70% in the inoculated zoysiagrass plants, for at least 5 weeks. Therefore, our protocol provides a fast and efficient toolbox for high-throughput functional genomics in zoysiagrass species, which may also have the potential to be applied to other warm-season turfgrass species.

8.2 Materials

8.2.1 Amplification of Target Gene

1. Zoysiagrass (*Zoysia japonica*) cultivar "Zenith" seeds (Pennington Seed Company, Madison, GA)
2. *Nicotiana benthamiana* seeds
3. Plastic nursery pots, trays, and tray covers
4. TRIzol reagent (Thermo Fisher Scientific, Waltham, MA)
5. TURBO DNA Free Kit (Thermo Fisher Scientific, Waltham, MA)
6. Microcentrifuge
7. Liquid nitrogen
8. Mortar and pestle
9. Chloroform
10. Isopropanol
11. Ethanol
12. High-Capacity cDNA Reverse Transcription Kit (Thermo Fisher Scientific, Waltham, MA)
13. Polymerase chain reaction (PCR) primers

14. GoTaq Master Mix (Promega, Madison, WI)
15. Thermal cycler
16. Gel electrophoresis system
17. Agarose
18. Ethidium bromide
19. TBE buffer (10X stock solution): 0.9 M Tris-borate, 20 mM EDTA, pH 8.0
20. DNA loading buffer (6X): 30% glycerol, 0.25% bromophenol blue, store at 4 °C.

8.2.2 Cloning Target Gene into Vector

1. PEG/$MgCl_2$ solution: 40% PEG 8000, 30 mM $MgCl_2$
2. TRV1 (19) and TRV2 vector (pYY13, Dong et al. 2007; Sha et al. 2014)
3. *Escherichia coli* cells (DB3.1 and DH5α)
4. Water bath or incubator
5. SOC growth medium (Thermo Fisher Scientific, Waltham, MA)
6. Incubating orbital shaker
7. Luria-Bertani (LB) medium: 10 g tryptone, 5 g yeast extract, 10 g NaCl. Bring to 1 L with pure water, adjust pH to 7
8. Kanamycin stock solution (50 mg/mL, 1000X): 0.5 g kanamycin. Bring to 10 mL with pure water, sterile filter, store at −20 °C
9. Petri dish
10. Resuspension buffer (P1): 50 mM glucose, 10 mM EDTA, 25 mM Tris (pH 8.0), store at 4 °C
11. NaOH/SDS solution (P2): 0.2 N NaOH, 1% SDS
12. Potassium Acetate solution (P3): 3 M KOAc (pH 6.0), store at 4 °C
13. Restriction nuclease: PstI and buffer (New England Biolabs, Ipswich, MA)
14. T4 DNA polymerase and buffer (New England Biolabs, Ipswich, MA)
15. dATP and dTTP
16. Dithiothreitol.

8.2.3 Agrobacterium Transformation

1. *Agrobacterium tumefacien* cells (GV3101)
2. Liquid nitrogen
3. Rifampicin stock solution (50 mg/ml, 1000X): 0.5 g rifampicin. Bring to 10 mL with pure water, sterile filter, store at −20 °C.

8.2.4 Inoculation

1. 10 mM $MgCl_2$
2. 10 mM MES (pH 5.6)
3. 200 μM acetosyringone
4. Seedlings of *N. benthamiana* and zoysiagrass "Zenith"
5. 1 mL needleless syringe
6. Silicon carbide (~400 mesh)
7. Scotch-Brite heavy duty scour pad
8. Latex gloves.

8.3 Methods

8.3.1 Preparation of Plant Materials

1. Sow seeds of *Z. japonica* cv. "Zenith" and *N. benthamiana* in plastic pots filled with soil (see Note 1). Set greenhouse/growth chamber parameters as follows: 28/25 °C day/night temperature, 60/70% day/night humidity, 200 μmol m^{-2} s^{-1} photosynthetically active radiation (PAR). Perform regular greenhouse irrigation and fertilization as needed. For the first week, trays need to be covered in order to facilitate seed germination.
2. A first batch of zoysiagrass "Zenith" plants can be prepared without *N. Benthamiana*, from which total RNA will be extracted.

8.3.2 Amplification of Target Gene Fragment (TGF)

1. Sequence fragments (around 200 ~ 400 bp) of target genes in zoysiagrass were identified from our *Z. japonica* genome assembly (unpublished data) (*see* Note 2).
2. PCR primers were designed in order to amplify TGF. To use ligation-independent reaction (LIC), LIC2 and LIC1 sequences were added in front of forward primer and reverse primer, respectively (Dong et al. 2007; Zhao et al. 2016).
3. RNA extraction using TRIZol reagent was performed for zoysiagrass plant leaf powder, which was first flash frozen in liquid nitrogen, and then ground using mortar and pestle.
4. Total RNA was treated with TURBO DNA Free kit to remove any genomic DNA contamination, and then was transcribed into cDNA using High-Capacity cDNA Reverse Transcription kit.
5. PCR amplification was performed to obtain TGF using cDNA as template.
6. Check the quality and quantity of PCR product by gel electrophoresis in a 1% agarose gel to ensure that it was specifically amplified and was the expected size.

7. Purify PCR product with the following procedures: (a) Add equal volume of PEG/$MgCl_2$ solution, PCR product and pure water, mix well; (b) Centrifuge for 20 min at 16,000 g; (c) Discard supernatant (*see* Note 3); (d) Resuspend the pellet in 70% ethanol; (e) Centrifuge for 20 min at 16,000 g; (f) Discard supernatant and air-dry the pellet for 15–20 min; (g) Dissolve the pellet with appropriate amount of pure water.

8.3.3 Cloning into TRV2-LIC Vector

1. Digest TRV2-LIC vector with PstI, and check digestion result by gel electrophoresis in a 1% agarose gel.
2. Set up T4 DNA polymerase reactions as described in Table 8.1. Mix well, and incubate at 22 °C for 30 min, followed by 70 °C for 20 min, and hold at 4 °C.
3. Set up LIC reaction as follows: Mix 5 μL of T4-treated vector and 5 μL of T4-treated PCR product together, incubate at 65 °C for 30 min, and decrease to 22 °C at 0.2 °C/s, and then incubate at 16 °C overnight (*see* Note 4).
4. Transform the LIC reaction product into *E. coli* competent cells. Thaw 100 μL of DH5α cells on ice, and then add LIC reaction product. Mix gently when pipetting to avoid agitation. Incubate on ice for 30 min. Heat shock cells for 30 s in a water bath at 42 °C and chill on ice for at least 2 min.
5. Add 1 mL of SOC growth medium, and shake for 1 h at 37 °C.
6. Centrifuge briefly to collect cells, and resuspend in 200 μL of LB medium. Spread cells on LB plates containing 50 μg/mL kanamycin. Incubate plates at 37 °C overnight.
7. Pick 3–5 colonies on the plates, and grow in 10 mL LB medium containing 50 μg/mL kanamycin until log phase.
8. Extraction of plasmid from E. coli. Collect cells from 1.5 mL E. coli culture by centrifuging for 2 min at 10,000 g. Discard supernatant and add 200 μL of ice-cold P1 solution, resuspend cells by vigorous vortexing (no cell clumps shall be observed). Add 200 μL of P2 solution, and briefly invert tube for 3–5 times until the solution becomes viscous and clear. Add 200 μL of ice-cold P3

Table 8.1 T4 DNA polymerase treatment

Component	Volume	Component	Volume
Digested TRV2 vector	2.50 μL (~50 ng)	Purified PCR product	2.50 μL (~50 ng)
10x NEB buffer 2.1	0.50 μL	10x NEB buffer 2.1	0.50 μL
100 mM dTTP	0.25 μL	100 mM dATP	0.25 μL
1 M DTT	0.05 μL	1 M DTT	0.05 μL
T4 DNA polymerase	0.10 μL	T4 DNA polymerase	0.10 μL
ddH_2O	1.60 μL	ddH_2O	1.60 μL
Total	5.00 μL	Total	5.00 μL

solution and briefly invert 5–8 times until no more precipitate accumulates, and solution becomes less viscous.

9. Centrifuge for 10 min at 15,000 g. Carefully transfer supernatant into a new tube by pipetting. Avoid any white precipitate to be transferred.
10. Add 2.5–3 volume of absolute ethanol pre-chilled at −20 °C, invert several times.
11. Centrifuge for 10 min at 12,000 g. Discard supernatant and air-dry pellet.
12. Dissolve pellet in appropriate amount of pure water.
13. The presence of TGF in TRV2-LIC vector can be verified by PCR and gel electrophoresis, as well as Sanger sequencing, using either gene-specific or vector-specific primers.

8.3.4 Preparation of Agrobacterium Used in VIGS

1. Transform TRV1, TRV2 (as empty vector control), and TRV2 containing TGF (TRV2-TGF) into *Agrobacterium tumefacien* strain GV3101. Thaw 100 µL of GV3101 cells on ice, add 50–100 ng TRV1, TRV2 or TRV2-TGF into cells by pipetting. Mix with pipetting and avoid agitation. Incubate on ice for 30 min.
2. Chill cells in liquid nitrogen for 5 min.
3. Heat shock cells in a water bath at 37 °C for 5 min. Chill on ice for at least 2 min.
4. Add 1 mL of SOC growth medium, and shake for 2 h at 28 °C.
5. Centrifuge briefly to collect cells, and resuspend in 200 µL LB medium. Spread cells on LB plates containing 50 µg/mL kanamycin and 50 µg/mL rifampicin. Incubate plate at 28 °C for at least 2 d.
6. Pick 3–5 colonies on the plates, and grow in LB medium containing 50 µg/mL kanamycin and 50 µg/mL rifampicin until log phase.
7. Extraction of plasmid from GV 3101 (*see* Sect. 8.3.3 Steps 8–12)
8. Confirm TRV1, TRV2 and TRV2-TGF constructs by either PCR or Sanger sequencing.
9. For long-term storage, add 500 µL of 50% sterile glycerol solution to 500 µL of log-phase GV3101 culture containing TRV1, TRV2 or TRV2-TGF vector, respectively. Mix well and store at −80 °C.

8.3.5 Agro-Infiltration of **N. Benthamiana**

1. One day before agro-infiltration, grow the transformed Agrobacterium GV3101 strains containing TRV1, TRV2, and TRV2-TGF respectively in 20 mL of LB medium with 50 µg/mL kanamycin and 50 µg/mL rifampicin overnight.
2. Harvest cells by centrifugation for 2 min at 10,000 g. Discard supernatant.
3. Resuspend cells in infiltration buffer containing 10 mM MES, 10 mM $MgCl_2$, and 200 µM acetosyringone and adjust to an OD600 of 1.0.

4. Incubate cell suspension for at least 3 h at room temperature.
5. Mix TRV1 and TRV2 (as empty vector control), TRV1 and TRV2-TGF in a 1:1 ratio, respectively.
6. Infiltrate *N. benthamiana* with cell mixture (TRV1 + TRV2, or TRV1 + TRV2 − TGF) into the abaxial leaf surface, using 1 mL needleless disposable syringe (*see* Note 5).
7. Transfer infiltrated *N. benthamiana* plants into a growth chamber with the following settings: 22/20 °C day/night temperature, 70% humidity, 200 μmol m^{-2} s^{-1} PAR to facilitate virus infection.
8. Leaves from TRV1 + TRV2 and TRV1 + TRV2 − TGF-infected *N. benthamiana* plants were harvested for subsequent inoculation into zoysiagrass. VIGS was monitored using a reporter gene (e.g. *PDS*) as positive control. Leaves from TRV1 + TRV2 − PDS-infected *N. benthamiana* plants that exhibited photobleaching phenotypes, indicative of suppression of the endogenous *PDS* gene, were used for inoculation into zoysiagrass.

8.3.6 Inoculation of Zoysiagrass

1. Collect VIGS-infected *N. benthamiana* leaves containing TRV1 + TRV2 and TRV1 + TRV2 − TGF, respectively.
2. Grind leaves using mortar and pestle, add a bit of silicon carbide (400 mesh) to facilitate abrasion during inoculation.
3. Cut Scotch-Brite heavy duty scour pad into thumbnail-size pieces. Dip one piece in leaf zap, and gently rub both sides of zoysiagrass leaf blade for five to eight times, with the direction from base to tip (*see* Note 6).
4. Cover inoculated zoysiagrass in the dark for 24 h at room temperature.
5. Remove cover and transfer zoysiagrass to growth chamber described in Sect. 8.3.5 Step 7.
6. Normally, the phenotype of VIGS will occur in 3 weeks.
7. Photograph the plants with silencing phenotype and collect tissues for subsequent experiments.

8.3.7 Evaluation of Gene Silencing

Here, we cloned a fragment of zoysiagrass *PDS* gene, as a reporter gene, into the TRV2 vector. The TRV1 + TRV2 − PDS-infiltrated *N. benthamiana* plants showed photobleaching phenotype, mostly on young leaves (Fig. 8.1). We then used photobleached leaf sap to inoculate zoysiagrass (Fig. 8.2). After three weeks, most of the

Fig. 8.1 Virus-induced gene silencing of the zoysiagrass *PDS* gene in *N. benthamiana* (Photos were taken at 14 d after agro-infiltration, left: control; right: silenced plant)

Fig. 8.2 Mechanical inoculation of zoysiagrass leaf blades with leaf sap collected from *N. benthamiana* plants expressing TRV1 + TRV2-*PDS*

newly developed leaf blades from inoculated zoysiagrass showed chlorosis phenotype (Fig. 8.3). RT-PCR analysis using primers corresponding to different regions of *PDS* gene confirmed the expression of the *PDS* gene in zoysiagrass was reduced up to 70%, at 5 weeks after inoculation (Fig. 8.4). Therefore, the test of *PDS* reporter gene in TRV-based VIGS system has validated our method as a fast and efficient tool for gene manipulation in zoysiagrass.

Fig. 8.3 Virus-induced gene silencing of the *PDS* gene in zoysiagrass (Photos were taken at 35 d after inoculation for empty vector control [left] and silenced zoysiagrass [right])

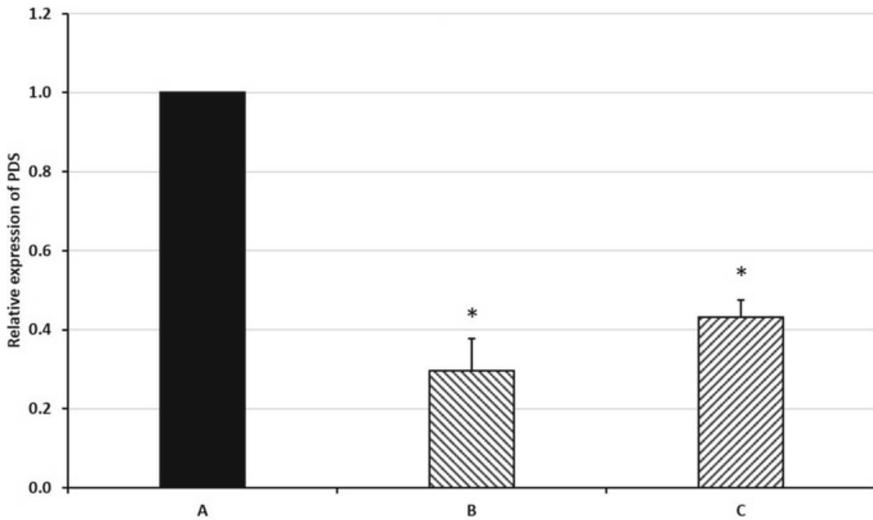

Fig. 8.4 RT-PCR analysis of expression of the *PDS* gene in silenced zoysiagrass plants (Data shown here are relative expression in zoysiagrass empty vector control [A], and two individually silenced plants [B and C]. The asterisk represents significant difference between control and VIGS plants, at the *P* level of 0.05)

Acknowledgements We thank Dr. Yule Liu (Tsinghua University) for providing the TRV vector. This work was supported by the National Institute of Food and Agriculture (NIFA)—Specialty Crop Research Initiative (SCRI) Grant 2015-51181-24291 and 2019-51181-30472 to QY, the USDA National Institute of Food and Agriculture Hatch Project TEX0-2-9374 to QY, and a start-up fund from the Texas A&M AgriLife Research and a Hatch Project from USDA National Institute of Food and Agriculture to JS (TEX0-1-9675).

Notes

1. In order to get ready for zoysiagrass inoculation, sow *N. benthamiana* seeds four weeks before zoysiagrass inoculation day, and zoysiagrass seeds three weeks before zoysiagrass inoculation day.
2. Use 3’ end of gene sequence in order to obtain more gene-specific fragments, however, avoid regions with high GC content or repetitive elements.
3. *Critical point*: be careful to remove the supernatant, since the DNA pellet is nearly invisible! It is recommended to use a pipettor to carefully aspirate it out. Same precaution applies to the rest of the purification steps.
4. The final step of incubation time at 16 °C could be shortened to 1 h, however, this may lead to decreased yield of ligation product.
5. It is recommended to choose the first four true leaves of *N. benthamiana* to infiltrate, since those leaves are thicker and juicier. Two-week old *N. benthamiana* plants usually have four true leaves, which are ideal for agro-infiltration.

6. *Critical point*: leaf age is of vital importance for successful VIGS assay. To ensure maximum VIGS efficiency, use "Zenith" seedlings that have three or less leaf blades. VIGS efficiency significantly decreases with older seedlings.

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Chapter 9
RNA Interference (RNAi): A Genetic Tool to Manipulate Plant Secondary Metabolite Pathways

Ashutosh R. Pathak, Swati R. Patel, and Aruna G. Joshi

Abstract Plants produce a variety of secondary metabolites which are being used as a source of medicine since the beginning of mankind, albeit most of them are synthesized in low concentrations. The developments in the field of 'omics' techniques help in the identification of genes of these metabolites having complex regulatory networks. Genetic engineering helps in manipulating the pathway which in turn increases the metabolite content and RNA interference (RNAi) is one such tool being used for the same. It is a homology dependent gene silencing technology in which the expression of pathway gene/promoter can be regulated by the introduction of double-stranded RNA (dsRNA) as it degrades the target mRNA. Since its discovery, this tool has been useful in manipulating the biosynthetic flux toward desired metabolite(s) by down-regulation of the competing pathway. In this chapter we discuss about RNAi as a tool to manipulate secondary metabolite pathways in plants.

Keywords Biosynthetic pathway · Medicinal plants · Metabolic engineering · RNA interference (RNAi) · Secondary metabolites

9.1 Introduction

Plants produce around 2,00,000 types of secondary metabolites as a defense response and they are useful sources of drugs, fragrances, pigments, food additives, and pesticides for mankind (Dixon and Strack 2003; Kutchan and Dixon 2005). It is estimated that 70–80% of the people worldwide rely mainly on herbal medicines for

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G. Tang et al. (eds.), *RNA-Based Technologies for Functional Genomics in Plants*,
Concepts and Strategies in Plant Sciences, https://doi.org/10.1007/978-3-030-64994-4_9

their primary healthcare (Canter et al. 2005). Reports document that out of 50,000–70,000 plants that are used worldwide for medicinal purposes, nearly 10,000 plants have become endangered (Brouwer et al. 2002; Edward 2004). World Health Organization (WHO) estimated that the market of herbal medicine will grow up to US$5 trillion by the year 2050 with an annual growth of 5–15% (Kumar and Gupta 2008). Due to complex chemical structures of the metabolites, they are difficult to synthesize chemically, and metabolites such as ajmalicine, ajmaline, artemisinin, berberine, colchicines, digoxin, ginsenosides, morphine, quinine, shikonin, taxol, vincristine, vinblastine, etc., are still extracted from plants (Rao and Ravishankar 2002). However plants synthesize metabolites in low concentrations and are restricted to a particular species or genus (Verpoorte et al. 2002). Thus to fulfill the demand, a large number of plants are collected from the wild which depletes the plants from natural habitat. Another problem faced by industries is the requirement of a large quantity of material for extraction of metabolites e.g., 2.5 kg of taxol requires 27,000 tons of *Taxus brevifolia* bark and thus the availability of plants for herbal medicines becomes a major problem (Rates 2001).

Synthesis of metabolites is under the control of different genes that are expressed in a particular tissue or cell type (Pichersky and Gang 2000). The plant genome contains 20,000–60,000 genes of which around 15–25% are involved in the synthesis of secondary metabolites (Bevan et al. 1998; Somerville and Somerville 1999). Metabolic engineering of pathways has key applications in alleviating the demands for limited natural resources (Lau et al. 2014). The secondary metabolite pathways are chain reactions catalyzed by enzymes that convert substrates into products with one or more branched points (Farré et al. 2014). Thus main challenge in manipulating the pathways is their complex nature which involves many regulatory factors (Kooke and Keurentjes 2012). Different strategies like blocking a competitive pathway, over-expressing regulatory genes/transcription factors, or inhibiting the catabolism of molecules can be used for the enhancement of metabolites (Koffas et al. 1999; Gomez-Galera et al. 2007).

9.2 Metabolic Engineering

Metabolic engineering is defined as the ‘directed improvement of product formation or cellular properties through the modification of specific biochemical reactions or the introduction of new genes with the use of recombinant DNA technology’ (Stephanopoulos 1999). The main aim of this technique is to redirect the precursor pool toward the synthesis of the desired compound(s) through alteration in the gene expression, and it is done either in positive (over-expression) or negative (down-regulation) manner (Pickens et al. 2011; Farré et al. 2014). The metabolic flux of the pathways can be regulated by the metabolites themselves, which in turn influences the activity of enzymes, transcription factors, and signaling proteins. The chemical diversity mainly arises through alkaloid, phenylpropanoid, and terpenoid pathways, thus number of studies have been carried out for identification of their regulatory

genes and transcription factors (Wu and Chappell 2008; Nagegowda 2010). High throughput ‘omics’ technologies like genomics, transcriptomics, proteomics, and metabolomics are being used for elucidation of the pathways (Vemuri and Aristidou 2005; Caspi et al. 2013). In non-model plants where whole genome sequencing is not available, gene identification is done by a comparatively cheaper technique like expressed sequence tags (ESTs) (Joshi and Pathak 2019). Thus, the process of metabolic engineering in medicinal plants research is divided into three steps: (i) selection of plant species and elucidation of the pathways through ‘omics’ technology, (ii) targeting the gene of interest through genetic engineering tool, and (iii) screening the plants for metabolite content (Lau et al. 2014) (Fig. 9.1).

One of the key ways to reduce the levels of undesirable metabolites is recessive gene disruption and dominant gene silencing (Tang and Galili 2004). But the latter is a more promising approach to decrease the synthesis of undesirable compounds by suppression of branch-point gene which redirects the enzymatic reactions to increase the metabolite(s) of interest (DellaPenna 2001). Silencing the expression of a particular gene can be done in three different ways: (i) transcriptional gene silencing (TGS), (ii) post-transcriptional gene silencing (PTGS), and (iii) translation inhibition (Hamilton and Baulcombe 1999; Mansoor et al. 2006). But the central dogma of life suggests that if mRNA is silenced, further synthesis of secondary metabolites will be stopped (Abdurakhmonov 2016). RNA interference (RNAi) also known as post-transcriptional gene silencing (PTGS) is frequently used for gene down-regulation and thus known as the ‘knock-down’ method (Tang and Galili 2004).

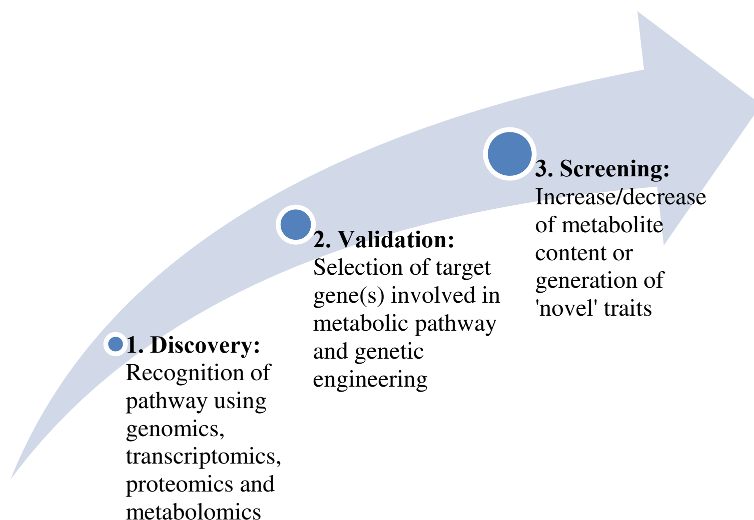

Fig. 9.1 Steps of metabolic engineering

9.3 RNA Interference (RNAi)

RNAi is a quick, easy, and sequence-specific homology-based tool to down-regulate the expression of targeted mRNA (Small 2007). Initially it was thought to function as a part of the defense mechanism against viruses when discovered in plants (Mansoor et al. 2006). The history of RNAi is nearly three decades old where Napoli and co-workers in 1990 transformed petunia plants with *chalcone synthase* (*CHS*) gene and the flower color changed from dark purple to white/chimeric, and this phenomenon was named as co-suppression. After five years, Guo and Kemphues (1995) reported knock-down of *par-1* gene expression in *Caenorhabditis elegans* through both sense and antisense RNA. The reason behind gene silencing remained unknown till Andrew Fire and Craig Mello reported that potent and specific genetic interference can be done by double-stranded RNA (dsRNA) in *C. elegans* which triggered the silencing of genes as it had identical sequences to the mRNA. This type of gene silencing was termed as 'RNA interference (RNAi)' (Fire et al. 1998) and in 2006 Fire and Mello received the Nobel Prize for discovering it (Allen et al. 2004). At the same time similar phenomenon was also reported in plants by Waterhouse et al. (1998) where dsRNA induced gene silencing which was more efficient than either sense or antisense RNA. RNAi technology suppresses the expression of enzymes that are expressed in the number of tissues at different developmental stages, whereas sense or antisense RNA fails to block the activity of enzymes that are encoded by multigene family (Larkin et al. 2007). Wesley et al. (2001) compared the silencing efficiency of hpRNA (dsRNA) and antisense RNA, and reported that hpRNA increases gene silencing by 90–100%. Thus it was confirmed that RNAi became the most promising tool for the suppression of dominant gene expression (Smith et al. 2000). One advantage of this tool is its dominant nature and the silenced gene is passed on in the T1 generation which created new opportunities in agriculture and production of metabolites (Lessard et al. 2002; Verpoorte et al. 2002). Many researchers use *in vitro* cultures to down-regulate the gene as it reduces the risk of contaminating food sources and environment, and provides a platform to test a metabolic engineering strategy that will be utilized for large scale production of metabolites (Wu and Chappell 2008). The main aims of RNAi technology for engineering secondary metabolites synthesis is given in Fig. 9.2.

9.3.1 Mechanism

Micro RNA (miRNA), short interfering RNA (siRNA), and small hairpin RNA (hpRNA) are types of small non-coding RNAs that are mainly involved in RNAi mechanism (Aukerman and Sakai 2003; Palatnik et al. 2003). Artificial microRNA (amiRNA)-based vectors have also proved to be effective for gene silencing since

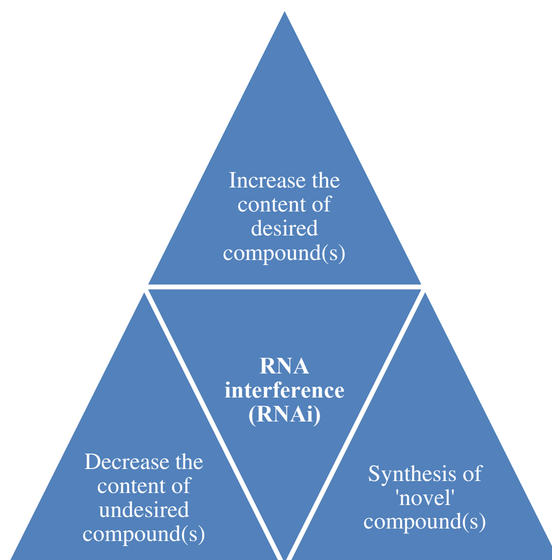

Fig. 9.2 Uses of RNAi technology in manipulating secondary metabolite pathway

the last decade (Warthmann et al. 2008). Smith et al. (2000) suggested that a more feasible approach is to clone both sense and antisense sequences separated by an intron region which forms a hairpin RNA (hpRNA) molecule upon transcription and triggers gene silencing. Aberrant single-stranded RNA (ssRNA) with an intron-hairpin construction triggers the generation of dsRNA by RNA-dependent RNA polymerase (RdRP) and activates the RNAi pathway (Waterhouse et al. 2001). Dicer, a ribonuclease III-type enzyme, is activated by ATP which recognizes dsRNA and cuts them into smaller segments of 21–25 bp. These small RNAs are then incorporated into a nuclease complex known as the 'RNA-induced silencing complex' (RISC) which contains argonaute protein (AGO). Then one of the strands of siRNA (guide strand) becomes stably associated with AGO and the other strand (passenger strand) is degraded. The guide strand then leads RISC to its target mRNA and AGO protein binds the guide strand to the target sequence for complementary base pairing. Successful docking of the RISC-siRNA complex with mRNA will then either block the translation or degrade mRNA using exonucleases (Kusaba 2004). Reports suggest that the directionality of dsRNA processing and the target RNA cleavage sites are predefined, and the sequence complementary to the guide siRNA will be recognized and cleave the target mRNA in the central region which is 10-12 nt from the 5' end of siRNA (Elbashir et al. 2001). Lastly, the siRNA molecules are amplified via RdRp on the target mRNA and these siRNAs will, in turn, induce a secondary RNA interference i.e., transitive RNAi (Denli and Hannon 2003).

9.3.2 Vector and Transformation Methods

Different vectors are used to suppress gene expression in plants and the vector-based RNAi technology was improved by using an intron as the linker (Waterhouse et al. 1998; Smith et al. 2000). These RNAi vectors are specifically designed to generate long dsRNA with the same sequence as the target genes. Similarly, vectors designed to express hairpin RNAs (hpRNAs) are also successfully applied to silence the corresponding target genes (Wesley et al. 2003). Nowadays biotechnology companies are developing specialized vector constructs for RNA interference in plants (see table), which after transformation into host plant converts into dsRNAs and triggers efficient silencing.

One of the major issues in plant genetic transformation is to obtain a stably transformed plant which depends on the transformation methods. The first choice is Gram-negative, soil-borne pathogen *Agrobacterium* spp., which is also known as 'natural genetic engineer' is commonly used to transform numerous dicotyledonous plants (Zupan et al. 2000). But the wild-type Ti plasmid is very large (200 kb) and difficult to manipulate, which was overcome by the development of binary vectors (Bevan 1984). In such a system, the Ti plasmid of *Agrobacterium* has been disarmed by removing the T-DNA and keeping *vir* regions intact. Simultaneously, a separate binary vector is constructed which carries an origin of replication that is compatible with the Ti plasmid of *Agrobacterium.* When the binary vector is introduced into *Agrobacterium* the *vir* genes of Ti plasmid will act *in trans* to transfer the recombinant T-DNA from the binary vector to the host plant cell. As the binary vectors are smaller and comparatively easier to construct than wild-type Ti plasmids, the *Agrobacterium*-mediated transformation is considered as a reliable technique (Lessard et al. 2002).

Transient gene expression in majority of the plant species can be done via particle bombardment and electroporation. These techniques are useful especially when long term expression is not required for e.g., to test the effectiveness of various gene constructs before stable transformation (Lessard et al. 2002). One of the advantages of this method is high transformation frequency, which resulted in the successful transformation of plastids in tobacco and tomato (Maliga 2001). But these methods require the use of tissue culture protocols to regenerate transgenic plants/callus whereas *Agrobacterium*-mediated transformation overcomes this limitation by directly transforming germ-line cells or seeds and is one of the first choice for RNA interference in plants (Tague 2001).

RNAi is a promising way to manipulate the metabolite pathway (Borgio 2009) and it was first used by Mahmoud and Croteau (2001) in *Mentha* x *piperita* to reduce the level of menthofuran through antisense suppression of the *mfs* gene which codes for the cytochrome P450 (+) menthofuran synthase, which in turn increased the content of essential oils in plants. Later on many studies documented that the content of various volatiles can be increased in *Mentha* spp. by silencing different genes or transcription factors (Mahmoud et al. 2004; Wang et al. 2016; Reddy et al. 2017). Since the beginning of this technique, *berberine bridge enzyme (BBE)* is the gene

of interest for RNAi research as many scientists knock-down the expression of this gene to study its effect on the content of different alkaloids, especially benzophenanthridine type in many plant species (Park et al. 2002; Frick et al. 2004; Fujii et al. 2007). Waterhouse et al. (1998) documented that this technology can be useful to alter the flower colors as compared to conventional breeding and genetic transformation. RNAi has been applied to suppress the genes of anthocyanin biosynthesis like *anthocyanidin synthase* (ANS) in *Torenia* spp. which changed the flower color in transgenic plants (Nagira et al. 2006; Nakamura et al. 2006). Similarly, other genes of flavonoid pathways like *isoflavone synthase (IFS)*, *flavone synthase II (FNSII)*, *flavonol synthase (FLS)*, *flavanone 3-hydroxylase (F3H)*, *flavonoid 3'-hydroxylase (F3'H)*, *flavonoid 3',5'-hydroxylase (F3'5'H)*, *flavone 6-hydroxylase (CYP82D1.1)*, *flavone 8-hydroxylase (CYP82D2)*, *chalcone isomerase (CHI)*, *chalcone synthase (CHS),* etc., were silenced and their effect on flavonoids was reported by many workers (Subramanian et al. 2005; Nakatsuka et al. 2007; Seitz et al. 2007; Park et al. 2011; Jiang et al. 2014; Zhang et al. 2015; Zhao et al. 2018). Recently Hu et al. (2020) reported that the down-regulation of one of the flavonoid biosynthetic pathway gene *laccase* gene *(Lac1)* affects the cotton fiber development. Whereas Liu et al. (2002) down-regulated the expression of two fatty acid desaturase genes i.e., *stearoyl-acyl-carrier protein Δ9-desaturase (SAD)* and *oleoyl-phosphatidylcholine ω6-desaturase (FAD)* in cotton seeds, which increased the content of stearic acid and oleic acid for better oil quality. Similarly, the content of different types of ginsenosides was increased or decreased in different species of *Panax* (*P. ginseng*, *P. notoginseng* and *P. quinquefolium*) by RNAi technique to identify the roles of different genes in ginsenoside biosynthetic pathway (Han et al. 2006; Zhao et al. 2015; Wang et al. 2017). This strategy has been used for commercial-scale production of desired plant products e.g., decaffeinated *Coffea arabica* and *Coffea canephora* plants were produced by silencing *theobromine synthase* gene using RNAi (Ogita et al. 2003, 2004). Table 9.1 depicts the plant species in which RNAi has been used to silence the secondary metabolite genes as well as the vector and transformation methods used for the same.

9.4 Conclusion

RNAi is the choice of present-day researchers to manipulate the genes synthesizing secondary metabolites. Since RNAi is a sequence-specific process, this requires the selection of a unique or conserved region of the target gene which ensures that the multiple gene families can be silenced. But the major bottleneck is that the complete information about the genomes of many non-model plants for secondary metabolite synthesis is lacking. The major drawback of RNAi tool is its unintended targets as 21–25 nt homology is required to suppress the gene function, even then it is still being used for identifying the gene functions and to increase the content of the desired metabolite.

Table 9.1 Down-regulation of plant secondary metabolite pathways using RNAi

Plant	Target metabolite(s)/ pathway	Target gene(s)/transcription factor	Vector*	Effect	Reference
Arabidopsis thaliana	Lignin	*Hydroxycinnamoyl transferase (HCT)*	pFGC5941[a]	Decreased content	Hoffmann et al. (2004)
	Flavonols, sinapate esters, and anthocyanins	*DNA-binding with-one-finger (DOF4;2)*	pJawohl8-RNAi[a]	Increased content	Skirycz et al. (2007)
	Putrescine	*ATR7*	pFGC5941[a]	Increased content	Sujeeth et al. (2020)
Artemisia annua	Artemisinin	*Cinnamate-4-hydroxylase (C4H)*	pART 27[a]	Increased content	Kumar et al. (2016)
	Artemisinin	*MYC2*	pHELLSGATE12[a]	Decreased content	Shen et al. (2016)
	Artemisinin	*Squalene synthase (SQS)*	pCAMBIA 2300[a]	Increased content	Ali et al. (2017)
	β-caryophyllene	*Pleiotropic drug resistance transporter (PDR3)*	pHELLSGATE12-iAaDPR3[a]	Decreased content	Fu et al. (2017)
	Artemisinin	*1-Deoxy-d-xylulose-5-phosphate reductoisomerase (DXR)*	pSGRNAi[a]	Decreased content	Wang et al. (2018)
	Artemisinin	*HY5*	pHELLSGATE12[a]	Decreased content	Hao et al. (2019)
Atropa belladonna	Tropane alkaloids (Hyoscyamine, scopolamine, and littorine), phenyllactate and tropine	1. *UDP-glycosyltransferase (UGT1)* and 2. *Littorine synthase (LS)*	pBin19[a]	Increased phenyllactate, tropine, and decreased hyoscyamine, scopolamine, and littorine content	Qiu et al. (2020)

(continued)

Table 9.1 (continued)

Plant	Target metabolite(s)/ pathway	Target gene(s)/transcription factor	Vector*	Effect	Reference
	Tropane alkaloid (hyoscyamine, anisodamine, and scopolamine), putrescine, and *N*-methylputrescine	*Ornithine decarboxylase (ODC)*	pBIN19[b]	Decreased content	Zhao et al. (2020)
Betula platyphylla	Betulin	*S-nitrosoglutathione reductase (GSNOR)*	pSGRNAi-GSNOR[a]	Increased content	Ma et al. (2019)
	Betulinic acid and oleanolic acid	1. *Cycloartenol synthase (CAS)* and 2. *β-amyrin synthase (β-AS)*	*pRNAi-GG (for CAS) and pCAMBIA 1303 (for β-AS)*[a]	1. Increased betulinic acid and oleanolic acid content 2. Increased betulinic acid and decreased oleanolic acid content	Yin et al. (2020)
Brassica napus	Pectin and lignin	*MYB43*	pFGC5941M-BnMYB43I[a]	Increased pectin and decreased lignin content	Jiang et al. (2020)
Eschscholzia californica	Benzophenanthridine alkaloids	*Berberine bridge enzyme (BBE)* and *N-methylcoclaurine 3'-hydroxylase (CYP80B1)*	pBI102[a]	Decreased content	Park et al. (2002)
	Benzophenanthridine alkaloid	*Berberine bridge enzyme (BBE)*	pBI102[b]	Decreased content	Park et al. (2003)
	(S)-Reticuline	*Berberine bridge enzyme (BBE)*	pART27[a]	Increased content	Fujii et al. (2007)

(continued)

Table 9.1 (continued)

Plant	Target metabolite(s)/ pathway	Target gene(s)/transcription factor	Vector*	Effect	Reference
Forsythia koreana	Pinoresinol and matairesinol	*Pinoresinol/lariciresinol reductase (PLR)* and *sesamin synthase (CYP81Q1)*	pSPB3104[a]	Increased pinoresinol and decreased matairesinol content. Novel sesamin synthesis	Kim et al. (2009)
Gentiana straminea	Oleanolic acid	*β-amyrin synthase (βAS)*	pK7GWIWG2D(II)[c]	Decreased content	Liu et al. (2016)
Glycine max	Isoflavone	*Isoflavone synthase (IFS)*	CAM-sUbi:GFP[b]	Decreased content	Subramanian et al. (2005)
	Saponins	*β-amyrin synthase (βAS)*	pUHR:7S-IR[c]	Decreased content	Takagi et al. (2011)
	Isoflavone	*Flavone synthase II (FNSII)* and *flavanone 3-hydroxylase (F3H)*	pCAMBIA3300[b]	Increased content	Jiang et al. (2014)
Gossypium hirsutum	Stearic acid and oleic acid	*Stearoyl-acyl-carrier protein Δ9-desaturase (SAD-1)* and *oleoyl-phosphatidylcholine ω6-desaturase (FAD2-1)*	pBI-Lec[a]	Increased content	Liu et al. (2002)
	Gossypol and pigmentation	*CGP1*	pHellsgate 4[a]	Decreased content	Gao et al. (2020)
	Flavonoids	*Lac1*	pHellsgate 4[a]	Increased content	Hu et al. (2020)
Isatis indigotica	Lignan (Conifer alcohol, lariciresinol, pinoresinol, secoisolariciresinol and its diglucoside and pinoresinol 4-O-glucopuranoside)	*WRKY34*	pCAMBIA1300-IiWRKY34[a]	Decreased content	Xiao et al. (2020)

(continued)

Table 9.1 (continued)

Plant	Target metabolite(s)/pathway	Target gene(s)/transcription factor	Vector*	Effect	Reference
Lavandula × intermedia	Essential oils	*1,8-cineole synthase (CINS)*	pBI121[a]	Change in essential oil composition/altered fragrance	Tsuro et al. (2019)
Linum usitatissimum	Lignin and phenolic acids	*Cinnamyl alcohol dehydrogenase (CAD)*	pHellsgate2[a]	Decreased content	Wróbel-Kwiatkowska et al. (2007)
Lithospermum erythrorhizon	Shikonin	*Ethylene insensitive3 like protein (EIL-1)*	pBI121[b]	Decreased content	Fang et al. (2016)
	Shikonin	*Multidrug resistance associated protein (MRP)*	pBI121[b]	Decreased content	Zhu et al. (2018)
Manihot esculenta	Cyanogenic glucoside	*Valine N-monooxygenase (CYP79D1* and *CYP79D2)*	pCAMBIA 2301[a]	Decreased content	Jørgensen et al. (2005)
Medicago truncatula	Apocarotenoids	*Carotenoid cleavage dioxygenases (CCD1)*	pRedRoot[b]	Decreased content	Floss et al. (2008)
Mentha × piperita	Essential oil	*Menthofuran synthase (MFS)*	pGAdekG[a]	Increased content	Mahmoud and Croteau (2001)
	Limonene	*Limonene-3-hydroxylase (LH)*	pGALS and pGALH[a]	Increased content	Mahmoud et al. (2004)
Mentha spicata	Monoterpenes	*YABBY5*	pK7WG2D[a]	Increased content	Wang et al. (2016)
	Monoterpenes	*MYB*	pK7WG2D[a]	Increased content	Reddy et al. (2017)
Mimulus lewisii and *M. verbenaceus*	Carotenoid	*Reduced carotenoid pigmentation2 (RCP2)*	pFGC5941[a]	Decreased content	Stanley et al. (2020)
Nicotiana benthamiana	Lignin	*Hydroxycinnamoyl transferase (HCT)*	pTV00[d]	Decreased content	Hoffmann et al. (2004)

(continued)

Table 9.1 (continued)

Plant	Target metabolite(s)/ pathway	Target gene(s)/transcription factor	Vector*	Effect	Reference
	Nicotine	*bHLH1* and *bHLH2*	pK7GWIWGII[a]	Decreased content	Todd et al. (2010)
	(+)-Valencene	*5-Epi-aristolochene synthase (EAS)* and *squalene synthase (SQS)*	pK7GWIWG2(II)[a]	Increased content	Cankar et al. (2015)
Nicotiana glauca	Nicotine and anabasine	*Ornithine decarboxylase (ODC)*	ODC-RNAi[b]	Decreased content	DeBoer et al. (2013)
Nicotiana tabacum	Anatabine	*Putrescine N-methyltransferase (PMT)*	pYC3JR[b]	Increased content	Chintapakorn and Hamill (2003)
	Diterpenes	*Cembratriene-ol cyclise (CYC-1)* and *CYP71D16*	pKYLX71–35S[2a]	Decreased content	Wang and Wagner (2003)
	Anthocyanins	*Chalcone isomerise (CHI)*	pEBisHR-35SintNtCHIir[a]	Decreased content	Nishihara et al. (2005)
	Nornicotine	*Nicotine N-demethylase (CYP82E4)*	pKYLX71[a]	Decreased content	Gavilano et al. (2006)
	Pelargonidin	*Flavonol synthase (FLS)* and *flavonoid 3'- hydroxylase (F3'H)*	pEBisBR[a]	Increased content	Nakatsuka et al. (2007)
	Nicotine	*Ornithine decarboxylase, (ODC), arginine decarboxylase (ADC) aspartate oxidase (AO), S-adenosylmethionine synthetase (SAMS), agmatine deiminase (AIC) arginase (ARG)*	p45-2-7-1[a]	Decreased content	Martinez et al. (2020)
	Flavonols, chlorogenic acid, and anthocyanins	*HDG2*	pHellsgate 2[a]	Decreased content	Wang et al. (2020)

(continued)

Table 9.1 (continued)

Plant	Target metabolite(s)/pathway	Target gene(s)/transcription factor	Vector*	Effect	Reference
Ocimum basilicum	Eugenol and coniferyl alcohol	*Coniferyl alcohol acyltransferase1 (CAAT1)*	pK7WG2D[a]	Decreased content	Dhar et al. (2020)
Panax ginseng	Ginsenoside	*Dammarenediol synthase (DDS)*	pK7GWIWG2(I)[a]	Decreased content	Han et al. (2006)
	Ginsenoside and phytosterol	*Squalene epoxidase1 (SQE1)*	pB7GWIWG2(II)[a]	Increased phytosterol and decreased ginsenoside content	Han et al. (2010)
	Ginsenoside Ro (Oleanane type)	*β-amyrin 28-oxidase (CYP716A52v2)*	pB7GWIWG2(II)[a]	Decreased content	Han et al. (2013)
	Ginsenoside (Protopanaxadiol and protopanaxatriol type)	*Protopanaxadiol synthase (CYP716A47)*	pBI121[b]	Decreased content	Sun et al. (2013)
	Ginsenoside and β- amyrin	*β-amyrin synthase (βAS)*	pK7GWIWG2(II)[b]	Increased dammarane-type ginsenoside and decreased β-amyrin and oleanane type ginsenoside	Zhao et al. (2015)
	Ginsenoside (Protopanaxadiol and protopanaxatriol type)	*Protopanaxadiol 6-hydroxylase (CYP716A53v2)*	pB7GWIWG2(II)[a]	Increased protopanaxadiol and decreased protopanaxatriol ginsenoside content	Park et al. (2016)
	Ginsenoside (Total, Rd and protopanaxadiol type)	*UDP-glycosyltransferase (3-O-UGT2* and *UGT94Q2)*	pBI121[b]	Decreased content	Lu et al. (2017b)

(continued)

Table 9.1 (continued)

Plant	Target metabolite(s)/ pathway	Target gene(s)/transcription factor	Vector*	Effect	Reference
Panax notoginseng	Triterpene and ginsenosides (Rb1, Rg1, Rh1, Re, and Rd)	*Cycloartenol synthase (CAS)*	pHellsgate2[a]	Increased content	Yang et al. (2017)
Panax quinquefolium	Ginsenoside	*Protopanaxadiol synthase (D12H)*	pBI121[b]	Decreased content	Sun et al. (2013)
	Ginsenosides (protopanaxadiol and protopanaxatriol type)	*Protopanaxatriol synthase (CYP6H)*	pBI121[b]	Increased protopanaxadiol and decreased protopanaxatriol ginsenoside content	Wang et al. (2014)
	Ginsenoside (Total, Rd and protopanaxadiol type)	*UDP-glycosyltransferase (3-O-UGT2* and *UGT94Q2)*	pBI121[b]	Decreased content	Lu et al. (2017b)
	Ginsenoside Rh2	*UDP-glucosyltransferase (3-O-UGT1)*	pBI121[b]	Decreased content	Lu et al. (2017a)
Panicum virgatum	Lignin	*Ferulate 5-hydroxylase (F5H) and caffeic acid O-methyltransferase (COMT)*	pANIC8D (for F5H) and pANIC8B (for COMT)[a]	Decreased content	Wu et al. (2019)
Papaver somniferum	*(S)*-Reticuline	*Codeinone reductase (COR)*	pPLEX X002i[a]	Increased content	Allen et al. (2004)

(continued)

Table 9.1 (continued)

Plant	Target metabolite(s)/ pathway	Target gene(s)/transcription factor	Vector*	Effect	Reference
	Alkaloids	*Berberine bridge enzyme (BBE)*	pPOP19[a]	Increased laudanine, reticuline, laudanosine, salutaridine, dehydroreticuline, scoulerine and decreased oripavine content in latex No change in benzophenanthridine alkaloids but decreased oripavine and salutaridine content	Frick et al. (2004)
	Salutaradine	*Salutaridinol 7-O-acetyltransferase (SalAT)*	pPLEX X002i[a]	Increased content	Allen et al. (2008)
	Morphine	*Salutaridinol 7-O-acetyltransferase (SalAT)*	pART27[a]	Increased salutaridine and salutaridinol and no change in morphine content	Kempe et al. (2009)
Petunia hybrida	Methylbenzoate	*Benzoic acid/salicylic acid carboxyl methyltransferase1* and *2 (BSMT1* and *2)*	pHANNIBAL[a]	Decreased content	Underwood et al. (2005)
	Volatile benzenoids	*ODORANT1 (ODO1)*	pK7GWIWG2(I)[a]	Decreased content	Verdonk et al. (2005)
	Phenylacetaldehyde and 2-phenylethanol	*Phenylacetaldehyde synthase (PAAS)*	pART27[a]	Decreased content	Kaminaga et al. (2006)

(continued)

Table 9.1 (continued)

Plant	Target metabolite(s)/ pathway	Target gene(s)/transcription factor	Vector*	Effect	Reference
	Benzoic acid, methylbenzoate, benzyl alcohol, and benzylaldehyde	*Benzoyl-CoA:benzyl alcohol/2-phenylethanol benzoyltransferase (BPBT)*	pCAMBIA 1303[a]	Increased benzyl alcohol and benzylaldehyde but decrease benzoic acid and methylbenzoate content	Orlova et al. (2006)
	Isoeugenol and other volatiles	*Coniferyl alcohol acyltransferase (CFAT)*	pFMV[a]	Decreased content	Dexter et al. (2007)
Pinus radiata	Lignin	*Hydroxycinnamoyl transferase (HCT)*	pAHC25[c]	Decreased content	Wagner et al. (2007)
Populus grandidentata × *P. alba*	Lignin	*p-coumaroyl-CoA 3-hydroxylase (C3′H)*	pART27[a]	Decreased content	Coleman et al. (2008)
Populus tremula × *P. alba*	Condensed tannins and salicinoids	*Tonoplast sucrose proton symporter (SUT4)*	pGSA1285[a]	No change	Harding et al. (2020)
Rehmannia glutinosa	Phenolic acids (Ferulic acid, caffeic acid, and chlorogenic acid)	*p-coumarate-3-hydroxylase (C3H)*	pRNAi-GG[a]	Decreased content	Yang et al. (2020)
Rosa hybrida	Delphinidin	*Dihydroflavonol 4-reductase (DFR)*	pSPB919[a]	Decreased content	Katsumoto et al. (2007)
Salvia miltiorrhiza	Rosmarinic acid and salvianolic acid B	*Phenylalanine ammonia lyase (PAL)*	pPAL1[a]	Decreased content	Song and Wang (2011)
	Danshensu and salvianolic acid B	*Cinnamoyl CoA-reductase (CCR)*	pART27-CCRi[a]	Increased content	Wang et al. (2012)
	Rosmarinic acid	*R2R3 MYB*	pART27[a]	Increased content	Zhang et al. (2013)

(continued)

Table 9.1 (continued)

Plant	Target metabolite(s)/pathway	Target gene(s)/transcription factor	Vector*	Effect	Reference
	Tanshinone	*Copalyldiphosphate synthase (CPS)*	pK7GWIWG2D (II)-SmCPS[b]	Decreased content	Cheng et al. (2014)
	Salvianolic acid B	*Cinnamoyl-coA reductase (CCR)* and *caffeic acid O-methyltransferase (COMT)*	pAtPAP1-SmCCRi-SmCOMTi[a]	Increased content	Zhang et al. (2014)
	Phenolic acids	*Chalcone synthase (CHS)*	pART27[b]	Increased content	Zhang et al. (2015)
	Tanshinone	*CYP76AH1*	pK7GWIWG2D-AH1[b]	Decreased content	Ma et al. (2016)
	Rosmarinic acid and salvianolic acid B	*Caffeoyl-CoA o-methyltransferase* (CCoAOMT)	pART27-CCoAOMTi[a]	Decreased content	Wang et al. (2017)
	Rosmarinic acid and salvianolic acid B	*Myeloblastosis (MYB)* and *transparent testa glabra1 (TTG1)*	p*SmTTG1*–RNAi and pMDC123SB-AtMIR390a-B/c[a]	Decreased content	Li et al. (2018)
	Phenolic acid	*bHLH51*	pSmbHLH51–RNAi[a]	Increased content	Wu et al. (2018)
	Tanshinone	*Ethylene response factor (ERF6)*	pCMBIA1304[b]	Decreased content	Bai et al. (2018)
	Tanshinone	*APETALA2/ethylene-responsive factor (AP2/ERF) (ERF128)*	pK7GWIWG2D (II)-GFP-SmERF128i and pK7WG2D-GFP-SmERF128OX[b]	Increased content	Zhang et al. (2019b)
	Tanshinone	*MYB98b*	pART27[b]	Decreased content	Liu et al. (2020)
	Phenolic acid and tanshinone	*bHLH92*	pK7GWIWG2D (II)[b]	Increased content	Zhang et al. (2020)
Scutellaria baicalensis	Baicalin, baicalein, and wogonin	*Chalcone isomerase (CHI)*	pK7GWIWG2[b]	Increased baicalein and wogonin, and decreased baicalin content	Park et al. (2011)

(continued)

Table 9.1 (continued)

Plant	Target metabolite(s)/pathway	Target gene(s)/transcription factor	Vector*	Effect	Reference
	Baicalin, baicalein, wogonin, and wogonoside	*Flavone synthase II* [(1) *FNS II-1*, (2) *FNS II-2* and (3) *cinnamic acid–specific coA ligase (CLL-7)*]	pK7WGIGW2R[b]	1. No effect 2. Decreased content and novel compound 3. Decreased content	Zhao et al. (2016)
	Baicalin, baicalein, wogonoside, and wogonin	1. *Flavone 6-hydroxylase (CYP82D1.1)* and 2. *Flavone 8-hydroxylase (CYP82D2)*	pK7WGIGW2R[b]	1. Increased wogonoside and wogonin, and decreased baicalin and baicalein content 2. Increased baicalin and decreased wogonoside and wogonin content No change in baicalein content	Zhao et al. (2018)
	Wogonin and wogonoside	*O-Methyltransferases (OMTs)*	pK7WGIGW2R[b]	Decreased content	Zhao et al. (2019)
Symphytum officinale	Homospermidine and pyrrolizidine alkaloids	*Homospermidine synthase (HSS)*	pH7GWIWG2(II)[b]	Decreased content	Kruse et al. (2019)
Taraxacum antungense	Chlorogenic acid and caffeic acid	*Hydroxycinnamoyl-CoA quinate hydroxycinnamoyl transferase (HQT)*	pCAMBIA1300[a]	Decreased content	Liu et al. (2018)
	5-Caffeoylquinic acid	*Hydroxycinnamoyl-coA quinate hydroxycinnamoyl transferase (HQT)*	pCAMBIA1300[a]	Increased content	Liu et al. (2019)

(continued)

Table 9.1 (continued)

Plant	Target metabolite(s)/ pathway	Target gene(s)/transcription factor	Vector*	Effect	Reference
Taraxacum koksaghyz	Rubber, poly(cis-1,4-isoprene), dolichols, inulin, and pentacyclic triterpenes	*cis-prenyltransferase-like 1 protein like subunit* 1. *CPTL1* 2. *CPTL2*	pLab12.5-pREF-TbRTA-RNAi (for *CPTL1*) and pFGC5951-TkCPTL2-RNAi (for *CPTL2*)[a]	1. Increased inulin and pentacyclic triterpenes but decreased rubber and poly(cis-1,4-isoprene) content 2. Decreased rubber but no change in poly(cis-1,4-isoprene), inulin and pentacyclic triterpenes content No change in dolichols content	Niephaus et al. (2019)
Torenia fournieri	Anthocyanin and chlorophyll	*Glutathione S-transferase (GST1* and 2*), ubiquitin conjugating enzyme (UBC), anthocyanidin synthase (ANS), putative cullin, putative flowering-time, glucose 6-phosphate/phosphate translocator (GPT), glutathione conjugate transporter*	pANDA35HK[a]	Decreased anthocyanin and variation in chlorophyll content	Nagira et al. (2006)
Torenia hybrida	Anthocyanidins	*Anthocyanidin synthase (ANS)*	pSPB[a]	Decreased content	Nakamura et al. (2006)

(continued)

Table 9.1 (continued)

Plant	Target metabolite(s)/ pathway	Target gene(s)/transcription factor	Vector*	Effect	Reference
	Aurone	*Aureusidin synthase 1 (AS1), flavanone 3-hydroxylase (F3H), chalcone 4-O-glucosyltransferase (4CGT), dihydroflavonol 4-reductase (DFR)*	pSFL308[a]	Novel color	Ono et al. (2006)
Tripterygium wilfordii	Triptolide	*Copalyl diphosphate synthases (TPS7v2* and *TPS9v2)* and *miltiradiene synthase (TPS27v2)*	pK7GWIWG2D[c]	Decreased content	Su et al. (2018)
	Triptolide and celastrol	*1-deoxy-D-xylulose-5-phosphate reductoisomerase (DXR)*	pK7GWIWG2D[c]	Decreased content	Zhang et al. (2018)
	Triptolide and wilforine	*3-Hydroxy-3-methylglutaryl- coA reductase (HMGR)*	pK7GWIWG2_II-RedRoot[b]	Decreased content	Zhang et al. (2019a)
	Triptolide, wilforgine and wilforine	*TGA1*	pYBA1132-RNAi[a]	Decreased content	Han et al. (2020)
Withania somnifera	Withanolide and sterol	*1-Deoxy-D-xylulose 5-phosphate reductoisomerase2 (DXR2)* and *3-hydroxy-3-methylglutaryl coA reductase2 (HMGR2)*	pART27[a]	Decreased content	Singh et al. (2014)
	Glycowithanolide, withaferin A and glycosylated sterols	*Sterol glycosyltransferases (SGTs)*	pFGC1008[a]	Increased withaferin A and decreased glycowithanolide content	Saema et al. (2015)
	Withanolide	*Cycloartenol synthase (CAS)*	pGSA1131[a]	Decreased content	Mishra et al. (2016)

(continued)

Table 9.1 (continued)

Plant	Target metabolite(s)/pathway	Target gene(s)/transcription factor	Vector*	Effect	Reference
	Campesterol, sitosterol, stigmasterol, and cholesterol	*Sterol methyltransferase1 (SMT1)*	pART27[a]	Increased cholesterol and decreased of campesterol, sitosterol, and stigmasterol content	Pal et al. (2019)

*Transformation method-
[a] *Agrobacterium tumefaciens*
[b] *Agrobacterium rhizogenes*
[c] Particle bombardment/Gene gun
[d] Virus

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Chapter 10
Improving Nutrient Value of Crops: Applications of RNAi in Targeting Plant Metabolic Pathways

Sarbajit Chakrabarti, Chanchal Chatterjee, and Arunava Mandal

Abstract The World Health Organization states that more than 820 million people worldwide do not have a regular supply of food grains. Considering the global hunger index, crop improvement becomes the only solution for combating global hunger. To alleviate the nutritional quality of crops, different techniques have been developed and applied by the researchers, namely conventional breeding approaches to genetic engineering approaches. RNA-based techniques to engineer metabolic pathways to increase the metabolic pool of the desired nutritionally important compounds have been in use. RNAi technique has emerged as one of the most successful means to silence the specific gene of interest in a tissue-specific manner to increase the metabolic pool of the desired compound. RNAi is successfully done to achieve biofortification, reduction of alkaloids, allergenicity, etc. In more recent times, CRISPR/Cas9 has emerged as a more sophisticated technique to engineer the metabolic pathway. Here, we have discussed how RNAi technology has been used in various spears of plant sciences for crop improvement.

Keywords Crop improvement · Metabolic engineering · RNA-based technologies · RNAi · Biofortification

10.1 Introduction

Plants are often regarded as the principal source of desired nutrients in human and livestock feed. The chances of the occurrence of type 2 diabetes, cardiovascular diseases, and cancer can be reduced by the consumption of fresh fruits and vegetables (Martin et al. 2011; Mozaffarian et al. 2011; Bradbury et al. 2014).

However, the incidence of malnourishment across the globe specially in developing and underdeveloped countries, has drawn the attention of the researchers and

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G. Tang et al. (eds.), *RNA-Based Technologies for Functional Genomics in Plants*,
Concepts and Strategies in Plant Sciences, https://doi.org/10.1007/978-3-030-64994-4_10

helped them to conclude that the crops consumed in the staple diet are failing to meet the daily dietary requirement. In this context, metabolic engineering can address this obstacle by specifically altering the metabolic pool of the staple crops to enhance nutritional content. Traditional breeding based on selecting genetic variations (natural or induced) or genetic engineering can be applied in redirecting the metabolic pathway but it has several limitations too, e.g. sometimes a given gene in its silenced form, though advantageous for rising seed quality, frequently leads to harmful effect on the plant itself (Negrutiu et al. 1984; Frankard et al. 1992). Therefore, targeted silencing of the gene by using tissue-specific promoter can help achieve the desired outcome without any unwanted deleterious effects. RNAi mediated by double-stranded RNA (dsRNA) is one such approach where a gene can be effectively silenced using specific promoters (Smith et al. 2000).

Here, in this chapter we are going to discuss why we need to improve the crops on nutritional perspective and how RNA-based technologies or more specifically RNAi, can help to reengineer the metabolic pathway, thereby improving the nutritional content of a food crop.

10.2 Crop Improvement

According to the World Bank Report of 2008, agriculture is an important sector known to play an important role in the attainment of developmental goals of any country. According to the 2018 report from WHO, globally 820 million population did not have enough food to eat, which was 811 million in the previous year. In developing countries, three out of every four people live in villages and the majority rely on agriculture directly or indirectly for their livelihood. Both developed countries and developing countries suffer from malnutrition, one through an inadequate supply of food, and in other by inappropriate choices, which is influenced by economic consideration. Beyond meeting the basic nutrition requirement, it is proven in several cases that the physiological process of an organism can be altered in any stage of life depending on the intake of food. Indeed, the majority of the population of the developing countries lacks vital minerals like iron, zinc, and iodine in their diet, which is the reason behind different types of health issues. Plant components of dietary interest fall into two categories—macronutrients (proteins, carbohydrates, and fibers), micronutrients (phytochemicals, vitamins, minerals, antinutrients, allergens).

Metabolic analysis of the plant metabolites provided a new way for more targeted crop improvement to meet the current need (Hall et al. 2008). Metabolic engineering has the potential to redirect one or more reactions to get better production of existing compounds, production of novel compounds, or to facilitate the degradation of detrimental compounds, thereby improving the nutritional quality of the crop. Thus crop improvement has a direct beneficial effect on mass health status (Table 10.1).

Table 10.1 List of genes manipulated in different plants for crop improvement through RNAi

Sr No.	Crop improvement	Traits improved	Gene targeted	Plant used	References
1.	Biofortification	β-Carotene	Lipoxygenase gene(r9 LOX1)	rice	Gayen et al. (2015)
		Lysine	LKR/SDH	Corn	Houmard et al. (2007)
		Iron	MIPS (myo-inositol-3-phosphate synthase)	soybean	Kumar et al. (2019)
		Amylose	SBEIIa and SBEIIb	wheat	Regina et al. (2006)
2.	Oilseed improvement	Reduction in linolenic acid (18:3) and increase in linolenic acid (18:2)	FAD3	soybean	Flores et al. (2008b)
		Oleic acid content	ghSAD-1 and ghFAD2-1	cotton	Liu et al. (2002)
3.	Hypoallergenicity	Reduction in Ara h 2 content	Ara h 2	peanut	Dodo et al. (2008a)
		Reduced content of Lyc e 3	Lyc e 3	Tomato	Lorenz et al. (2006)
		Reduced content of Mal d1	Mal d1	Apple	Gilissen et al. (2005a)
		Reduced content of linamarin and lotaustralin	CYP79D1 and CYP79D2	Cassava	Siritunga and Sayre (2003)
		Reduced content of nornicotine	*N*-dem-ethylase	Tobacco	Gavilano et al. (2006)

10.3 Metabolic Engineering

The plant metabolic engineering can be defined as an attempt to engineer the endogenous metabolic pathways operating in a plant and redirecting one or more enzymatic reactions in the metabolic pathway for producing a new compound, facilitating the degradation of compounds, or improving the production of existing compounds. To achieve this, a methodical understanding of metabolic pathways is necessary. Metabolic pathways can be visualized as a string of linear, cyclical three-dimensionally arranged interlinked enzymatically catalyzed chemical reactions where substrates are converted into products through a series of intermediates. These reactions maybe unidirectional or reversible and there may be several branch points. In plants particularly, the metabolic pathway seems to become increasingly complex as often there is a need for metabolic intermediates to transport between subcellular compartments or sometimes among the cells.

10.3.1 Generation of End Product of Metabolic Pathway

The simplest objective of metabolic engineering is to increase the end product. Here the researchers work on the options to increase the availability of upstream precursors or decrease the activity of rate-limiting enzymes to enhance the accumulation of end product. For example, metabolic engineering approaches targeted for a simultaneous increase in metabolic flux through the pterin and PABA branches help to enhance folate levels (De La Garza et al. 2007; Storozhenko et al. 2007). Recent studies revealed that the co-expression of GTPCHI (GTP cyclohydrolase I) and ADCS (aminodeoxychorismate synthase) in transgenic tomato fruit results up to 25-fold higher expression levels of folate in transgenic fruits than those of wild-type fruits (De La Garza et al. 2007).

10.3.2 Accumulating an Intermediate Product

The intermediates in any metabolic pathway have a tendency to get converted into end products, therefore modulating the accumulation of the intermediate product is difficult. To overcome such a challenge, redirecting the metabolic strategies focus on a combinatorial approach to boost up the upstream flux and block downstream flux through competitive pathways, or creation of metabolic sinks to remove intermediates and to avoid their additional conversions. Carotenoid pathway can excellently demonstrate this approach because several intermediates of this pathway, e.g. zeaxanthin, β-carotene, and lycopene are essential for nutrition and focus of the engineering

approaches concentrate on increasing the accumulation of specific carotenoid intermediates. The silencing of CHY1 and CHY2 genes through RNAi in potato tubers resulted in increased β-carotene levels compared to the wild type (Diretto et al. 2007).

This example clearly demonstrates how blocking the downstream steps can block the loss of a specific intermediate. Another strategy often employed is the diversion of the intermediate into a subcellular compartment, lacking the enzymes required for additional conversion, and at the same time creation of a metabolic sink which can shift the equilibrium toward production. For example, *Orange* (*Or*) which is a gain-of-function mutation in cauliflower results in the differentiation of proplastids or non-colored plastids into chromoplasts, suggesting the fact that the formation of a metabolic sink for sequestering carotenoids can support carotenoid gathering in plants. The expression of *Or* gene of cauliflower in potato under a tuber-specific promoter renders orange-yellow color and increased accumulation of carotene in the transgenic potato compared to the wild type (Lopez et al. 2008).

10.3.3 Strategies to Alleviate Several Compounds Simultaneously

Sometimes in a metabolic pathway, multiple intermediates appear to be nutritionally essential. In such cases, the approaches for boosting flux through a pathway generally leads to an enhancement in the accumulation of end products, but at the same time inhibiting upstream flux may backfire as there are chances of other essential molecules getting depleted. To overcome such a challenge, the most accepted strategy is to progressively restrict the metabolic flux in such a way that the early rate-limiting steps are removed but the competence of succeeding reactions gets gradually reduced, to allow the gathering of specific intermediate compounds. Transgenic maize plants not only accumulate high levels of β-carotene but also contain higher levels of lutein and zeaxanthin compared with wild-type plants, revealing the applicability of this approach (Naqvi et al. 2009).

Nowadays metabolic engineering is one of the most viable ways to increase the levels of different vitamins and intermediate products in plants.

10.4 Tools for Metabolic Engineering and RNA-Based Technologies as a Promising Approach

The decoding of the genome sequences of many crops made it possible to identify many genes, those encoding plant secondary metabolites. This opens the possibility for proper utilization of plant resources. It also makes it possible to overproduce and isolate the valuable plant-derived chemicals from new tailored systems that are being developed.

10.4.1 Development of Customized Systems for Overproduction of Plant Products

Plant metabolic pathways can be monitored in a heterologous organism by the introduction of genes encoding the pathway enzymes. *Botryococcus braunii* is a green alga responsible for the production of linear branched-chain triterpenes, which is increasingly being recognized as an important chemical and biofuel (Hillen et al. 1982). However, large-scale production and isolation of these compounds are unrealistic due to the slow-growing nature of *B. braunii*. Recently, triterpene botryococcene were produced in *N. tabacum* plants by the overexpression of an avian farnesyl diphosphate synthase in the chloroplast (Jiang et al. 2016).

10.4.2 Engineering New Traits into Crops

As the cultivable land is decreasing gradually, more attention is required to increase the nutritional value of the crops that are grown. Tomato has recently undergone some engineering efforts for the improvement of the production of various metabolites (Butelli et al. 2008; Augustine et al. 2013; Giorio et al. 2013; Gutensohn et al. 2014). In very recent times, phenylpropanoid production has been upregulated in tomato fruits by introducing *At*MYB12 transcription factor under the regulation of a fruit-specific promoter.

10.4.3 Genetic Approaches

In the context of the metabolic engineering, genetic approach is another way, mostly studied in maize. But its success is relatively limited to other crops, typically due to the inadequate availability of genetic resources. In the mid of the twentieth century, an initiative was taken for the identification of high lysine content varieties in maize through the genetic approaches, to overcome the low Lys content in maize. However, with this effort high Lys *opaque2* mutant was discovered (Mertz et al. 1964). But the *opaque2* mutant still faces the problem with storage and transportation due to presence of soft kernel, making these tasks quite difficult. However, it was possible to overcome this barrier by selecting quantitative trait loci (QTL) which can restore kernel hardness in the presence of *o2*. Through developing a variety called Quality Protein Maize (QPM) and by targeting g-zeins suppression, without the *o2* mutant, rebalance of the Lys content and a vitreous kernel phenotype was maintained successfully (Planta and Messing 2017). To overcome the problems of rancidity and decreased shelf-life of soybean oil products, researchers have come up with genetic approaches. They were successful in establishing low linolenic acid soybean oil (low-lin) with the help of mutational breeding (Hammond and Fehr 1983; Wilcox and Cavins 1985; Fehr et al. 1992).

10.4.4 RNA-Based Technologies as an Emerging Approach

Though the traditional breeding technique has tremendous success in improving the dietary content of food and feed (Davies 2003), there is little success and the process is extensive, time consuming, and most crop plants have few available genetic resources. Among the many causes for the inadequate genetic resources available for breeding (Hoisington et al. 1999), two most significant reasons are (i) reduced gene pool through the domestication and breeding of crop plants (Lee 1998), and (ii) the genes that may be advantageous in one plant might have deleterious effect on other plants (Negrutiu et al. 1984; Frankard et al. 1992). Genetic engineering as well as gene transfer technologies became more popular due to the drawbacks of the conventional breeding approaches. Directed efforts by genetic engineering of metabolic pathways has led to altered plant nutrients (Galili 2002). These efforts need a comprehensive understanding of the constituent enzymes and plant metabolic pathways. The main strategy to enhance plant nutrients is by escalating the expression of anabolic biosynthetic genes but the two main reasons limiting the efficacy of this approach are (i) extra gene copy introduction may have the nonintuitive effect which may diminish the expression from both the introduced as well as homologous endogenous loci—this incident of gene silencing is recognized as co-suppression (Napoli et al. 1990) (ii) maintenance of homeostatic level of nutrients, which are tend to be controlled by feedback metabolic loop. The main objective of recent technologies is to attain loss-of-function phenomenon by specific targeting of a gene. To achieve loss-of-function, DNA or RNA of a particular gene can be targeted generating gene alteration or silencing. Specific gene in the genome can be targeted by technologies like zinc-finger nuclease (ZFN) (Urnov et al. 2010), transcription activator-like effector nuclease (TALEN) (Zhang et al. 2013), mega-nucleases, and clustered regularly interspaced short palindromic repeats (CRISPR)/CRISPR-associated nuclease9 (CRISPR/Cas9) system (Xie and Yang 2013). In transgenic plants, tissue-specific manipulation is of great significance not only for overexpression but also for silencing. Indeed, RNAi technology has the huge potential to engineer the nutritional value of plant organs in a more precise way. Seed-specific RNAi approach has been utilized fruitfully to suppress the expression of maize zein storage protein, leading to the generation of dominant high lysine corn (Segal et al. 2003).

10.5 Mechanism of RNAi

Scientists have demonstrated RNAi related phenomenon in plants much before the discovery of it as a gene silencing event, in worm by Guo and Kemphues (Kusaba 2004). One such phenomenon is co-suppression, where a sense transgene mediates gene silencing. Here, upon introduction of the exogenous transgene, a coordinated silencing of transgene and its endogenous homolog (or homologs) was observed. Co-suppression can be further divided into transcriptional gene silencing (TGS)

and post-transcriptional gene silencing (PTGS). This PTGS is also termed as RNA interference or RNAi. RNAi is an evolutionary-conserved biological mechanism that mediates sequence-specific gene regulation, as the introduction of double-stranded RNA results in the prevention of expression of specific genes, i.e. silencing of the genes before they get translated.

10.5.1 Generalized Strategy of RNA Interference

Long dsRNA enters into the cell and turn on the RNA interference mechanism of the cell. In the series of events, first, the dicer enzyme is recruited followed by cleavage of dsRNA into small interfering RNA (siRNA). Next, the two strands of siRNA get distinguished between sense and antisense strands based on the similarity of sequence between the siRNA strand and the gene to be targeted. For example, a sense strand denotes the strand of siRNA having the sequence exactly similar to the target gene. This phenomenon is assisted by RNA-Induced Silencing Complex (RISC). Subsequently, sense strands get degraded, whereas the antisense strand gets incorporated into RISC and functions as a guide for the target messenger RNA (mRNA) in a sequence-specific way. RISC next cleaves mRNA and activated RISC can take part in the degradation of mRNA in a repeated manner.

10.5.2 Pathways Operating in Plants for RNA Silencing

MicroRNAs (miRNAs) are a type of small RNAs which are a part of PTGS. But their biogenesis is different from the siRNAs, therefore miRNA operating in RNA silencing pathway in plants requires a different design of transgene. The primary miRNA transcripts, also known as primary-miRNA or pri-miRNA, are synthesized by RNA polymerase II and undergo processing to produce mature miRNAs. Dicer cleaves the miRNA precursors and yields the mature miRNA (Grishok et al. 2001; Hutvágner et al. 2001; Lee et al. 2002; Reinhart et al. 2002; Kim 2005). miRNA-miRNA* forms duplex, which is subsequently methylated by HEN1 (Boutet et al. 2003). The duplex then, gets unwound and mature miRNA binds to argonaute (endonuclease with PAZ, MID, and PIWI domains) and the passenger strand gets degraded or rejected. This leads to the formation of miRNP complex. Now as the sequence of miRNA is only partially complementary to 3’UTR of target mRNA, AGO protein fails to cleave it. miRNA then interacts with target RNA and mediates RNAi effect via (1) Repressing mRNA translation, and (2) Removal of mRNA poly (A) tail.

10.5.3 Transformation Methods for RNAi Constructs into Plants

There are several plant-specific RNAi vectors that produce self-complementary dsRNA when expressed in plants (Horiguchi 2004). Different research laboratories have reported different types of constructs that have the ability to induce PTGS with nearly 100% effectiveness (Smith et al. 2000). Researchers have widely used the pANDA vector to introduce the RNAi effects in plants via *Agrobacterium*-mediated transformations (Miki and Shimamoto 2004). Occasionally, particle bombardment (Panstruga et al. 2003) and electroporation (Akashi et al. 2004) are also used for expressing RNAi cassettes in plant cells.

10.6 Applications of RNAi

10.6.1 Biofortification

"Biofortification" can be understood as approaches for increasing the dietary value of food crops and at the same time increasing the bioavailability of the nutrients to the population. Indeed, with the aid of modern biology techniques, plant breeding approaches it is possible to develop biofortified crop plants. In today's world, malnourishment and hidden hunger have appeared as a burning issue, mostly in the developing countries (Muthayya et al. 2013; McGuire 2015) which signifies that in the daily diet intake, essential micronutrients are not adequate. Therefore, nowadays researchers, across the globe, are focusing on shifting the research perspective from increasing the productivity of food crops to generating food crops sufficiently rich in nutrient value. Although nutrient supplementation can be a solution to address this undernutrition problem, it has several inherent limitations (Gilani and Nasim 2007; Pérez-Massot et al. 2013).

In this context, biofortification can be a long term and sustainable solution as it can provide a balanced diet having food crops rich in micronutrients (Hirschi 2009). Humans need approximately 40 micronutrients to have physical and mental development (White and Broadley 2005). Some of these are iron, manganese, copper, zinc, iodine, and vitamin A. Essential nutrients like iron, vitamin A, calcium, iodine are not present in sufficient amounts in rice, wheat, cassava, and maize. Hence, the population that strictly depends on these agricultural products fails to meet the daily requirement leading to sickness, morbidity, and stunted growth (Branca and Ferrari 2002).

Therefore, biofortification approach focuses to meet the energy needs and provides a diet with all essential nutrients by enhancing desired nutrient content in the edible portion of the crop to be consumed in the diet (Welch and Graham 2005).

10.6.1.1 RNAi in Enhancing Lysine Content

The inability of humans and cattle to synthesize lysine renders it as an essential amino acid. Population is dependent upon crops consumed as foods to meet their demand for lysine. In plants, 3-aspartic semialdehyde, derived from aspartate, functions as the point where metabolism gets directed toward the lysine branch, diverging from methionine, threonine, and isoleucine biosynthesis (Azevedo et al. 2006; Stepansky et al. 2006). The biosynthesis of lysine from aspartate, involves two key enzymes viz. dihydrodipicolinate synthase (DHDPS) which catalyzes the first committed step for lysine production and lysine-ketoglutarate reductase/saccharopine dehydrogenase (LKR/SDH), a bifunctional enzyme, involved in lysine catabolism. The accumulation of lysine is thought to contribute to feedback inhibition of DHDPS and forward activation of LKR/SDH (Azevedo et al. 2006; Stepansky et al. 2006). To improve the lysine content of crops, researchers across the globe are mostly focusing on these two key steps of lysine metabolism.

10.6.1.2 Corn

Corn is a worldwide harvested and largely consumed crop. Though corn grain is rich in carbohydrate, protein, and oil, one major drawback is the lower level of essential amino acid lysine in corn grain, which limits its nutrient value (Houmard et al. 2007). Researchers have identified that RNA interference-mediated silencing of LKR/SDH alone resulted in suppression of lysine catabolism in maize endosperm. However, it leads to accumulation of free lysine in mature kernels. In their experiment, they constructed the transgenic cassette by using the maize endosperm-specific b32 promoter, Adh1 intron, and Glb1 terminator along with the IR sequence targeting ZLKR/SDH. *Agrobacterium*-mediated transformation method was used to introduce the cassette in maize embryo, further Western blot analysis revealed reduced accumulation of LKR/SDH protein in transgenic plants.

F2 plants were obtained from self-pollinated hemizygous F1 and further advanced to F3 generation. Up to 20-fold increase in free lysine content was observed in transgenic F3 plants than the control plants.

Therefore this study represents the application of RNAi to develop transgenic crops with high lysine content. Other group of researchers transformed maize with constructs expressing chimeric dsRNA, and their results showed kernels with significant declines in the both 19- and 22-kD a-zeins accumulation, resulting in high content of lysine and tryptophan levels in transgenic maize compared to wild type (Huang et al. 2006).

10.6.1.3 Iron Biofortification in Crop by Phytate Reduction Using RNAi as a Tool

Worldwide, nearly 2 billion people are suffering from iron deficiency along with other mineral and vitamin deficiencies (Mayer et al. 2008). According to the report of WHO 2015, the pregnant women and the preschool students are worst sufferers due to lack of iron in the diet. Through the intake of diversified foods, micronutrient supplements, and medicines, the condition can be improved easily (Morrissey and Guerinot 2009) but the limitations such as geographical and financial capabilities make them unavailable to everyone (Mayer et al. 2008). The bioavailability of iron in crops can be increased by agronomic practices, conventional breeding, and genetic engineering, more successfully by targeting the gene of interest.

Some food like maize, rice, soybean, contains antinutrient like phytic acid (PA), which chelates metal ions like iron, zinc, which render them insoluble (White and Broadley 2005). The hydrolysis of phytic acid is catalyzed by the phytase enzyme, which subsequently leads to the release of phosphate and minerals (Welch and Graham 2005). Monogastric organisms lack microbial phytase in their gut, making them incapable to remove phosphate from myoinositol, thereby reducing the iron bioavailability. Thus, the manipulation of the phytate level is a real move toward nutrient content in crops.

Though soybean is one of the most nutritious and economically important food crop, it is accredited with high level of PA, where 2% of the total seed dry weight is accounted by PA. PA biosynthesis is a multistep process involving many enzymes. MIPS (myo-inositol-3-phosphate synthase), which catalyzes the primary as well as the rate-limiting step and IPK1 (Inositol 1, 3, 5, 6-pentakiphosphate 2-kinase) acts in the final step (Ali et al. 2013).

MIPS catalyzes the formation of inositol-6-phosphate from glucose 6-phosphate followed by sequential phosphorylation at the remaining five positions of the inositol ring in an ordered manner through various enzymes. It is evident that RNAi is an efficient technique to effectively downregulate PA synthesis genes in rice, wheat, and soybean. Many researchers successfully attempted to silence the MIPS genes by RNAi approach. Transgenic soybean plants have been generated carrying RNAi constructs and silencing MIPS under a seed-specific promoter, vicilin. In this attempt they have generated a MIPS1 intron hairpin construct, expressed using vicilin promoter, and transformed in normal seedlings of soybean through Agrobacterium-mediated transformation. In those transgenic plants, about 42% reduced phytate content with an increased iron bioavailability of 77% were observed.

10.6.1.4 Application of RNAi in Enhancing Beta-Carotene Content of Crops

Carotenoids have antioxidant activity which reduces the occurrence of cancers and other photosensitive diseases. Thus, carotenoids play various roles in human nutrition and provide many biological functions (Takemura et al. 2014). Human beings particularly depend on edible crop sources to fulfill the dietary requirements of carotenoids. Vitamin A is derived from provitamin A which is also known as beta-carotene. Deficiency of vitamin A can result in night blindness, xerophthalmia, measles, etc. (Paine et al. 2005; Parkhi et al. 2005; Guzman et al. 2010; Shumskaya and Wurtzel 2013). Nowadays vitamin A deficiency is a major problem as huge numbers of people are suffering from this deficiency (Tang et al. 2009). To obtain this beta-carotene, people mostly depend on animal products or on plant products like dark-green leafy vegetables and fruits. Rice is mostly consumed in the developing countries. However, rice lacks beta carotene, hence researchers have developed beta-carotene rich golden rice by engineering the carotinoid biosynthetic pathway (Ye and Beyer 2000; Datta et al. 2003; Paine et al. 2005).

Another major challenge for the production of biofortified rice is to maintain the dietary qualities of the carotenoid-enriched transgenic rice.

Previous studies have revealed that beta-carotene is more prone to oxidation owing to the existence of conjugated double bond system (Leenhardt et al. 2006). It is also an established fact that r9-LOX1 gene of rice is involved in quality deterioration of seed during storage (Gayen et al. 2014). Therefore, several groups have tried to develop transgenic golden rice by silencing the endogenous lipoxygenase gene through RNAi approach with the motive to reduce co-oxidation of carotenoids (Gayen et al. 2015). Gateway technology-based RNAi vectors were used to reduce the LOX activity in rice seeds, utilizing tissue-specific oleosin-18 promoter. Oleosin-18 promoter was chosen as it was capable of reducing LOX activity particularly in the embryo and aleurone layer of rice grains. The constructed RNAi vector was next introduced into golden rice line by PDS-1000/He particle delivery system. The enzyme assay revealed that LOX-RNAi transgenic golden rice seeds (T_3) have reduced LOX activity, consistent with significant reduction in mRNA expression level of LOX gene in different transgenic lines (T_3). Data obtained from HPLC analysis after artificial storage clearly demonstrated that downregulation of LOX reduces degeneration of carotenoids at storage condition. The LOX-RNAi seeds produced less significant amount of ROS than control seeds during storage condition.

This negligible generation of ROS in LOX-RNAi seeds suggests that *LOX* had undergone sufficient downregulation in those transgenic golden rice lines. All these findings cumulatively help to conclude that downregulation of lipoxygenase enzyme activity decreases depletion of carotenoids of biofortified rice seeds, suggesting it to be a possible way to enhance the storage stability and reduce huge postharvest losses of biofortified rice.

10.6.2 High Amylose Starch Production by RNAi

The main cause of mortality is coronary heart disease, cancers, and diabetes which are somehow associated with the diet of an individual. Indeed, to avoid these kinds of chronic diseases the important strategy would be to improve the nutritional quality of the foods that are frequently consumed. Wholegrain cereals, like wheat, are prime targets because of their well-established nutritional candidature and health-promoting prospective. Human beings obtain maximum minerals, proteins, and fibers from wheat. As wheat is considered as the staple food for majority of the population, it can be considered as an important tool to bring about changes in dietary composition by manipulating the grain composition. The major components of dietary fiber are resistant starch as well as non-starch polysaccharides those act significant role in improving health-related issues. In starch, glucose molecules are polymerized through α-1, 4 and α-1, 6 linkages to produce two classes of polymers—amylose and amylopectin. Amylose molecules in cooked food reassociate rapidly and form a complex that resists digestion. However, reassociation of amylopectin is slow and thus digests rapidly, which explains the presence of higher resistant starch in high amylose products.

Amylose is synthesized by ground-branching enzymes (GBSS), whereas amylopectin requires a complex pathway involving several isoforms of SS, starch-branching enzyme (SBE), and starch-debranching enzymes (Ball and Morell 2003). The isoforms of SBE are different for monocots and dicots. Being a monocot crop, maize has three isoforms of SBE, SBEI, SBEIIa and SBEIIb. The suppression of SBEIIa gene in maize led to induction of amylase content from 50 to 90%, but there is no such impact on amylase content upon silencing of SBEI and SBEIIa genes (Blauth et al. 2002). High amylose phenotype (>70%) was observed as a result of silencing of SBEIIa and SBEIIb genes. In this method, DNA fragments corresponding to exons 1, 2, and 3 and intron 3 of wheat SBEIIa gene and SBEIIb gene were ligated to generate SBEIIa and SBEIIb RNAi construct. The ligated sequence was introduced into an intermediate vector with promoter sequence from wheat (Anderson et al. 1989) along with terminator sequence of nopaline synthase gene from Agrobacterium (Depicker et al. 1982). The expression cassette from intermediate vectors was then introduced into binary transformation vectors to make hp-SBEIIa and hp-SBEIIb construct which was transformed into wheat through *Agrobacterium tumefaciens.*

10.6.3 RNAi in Oilseed Improvement

Vegetable oils are basically triacylglycerols in which three fatty acids are attached to a glycerol backbone. Therefore, they are widely present in human and livestock nutrition as an important source of high calorie. However, in addition this vegetable source also contributes to the growth of a variety of industrial chemical production, e.g. biodiesel (Durrett et al. 2008; Dyer et al. 2008). But the problem associated

with this vegetable oil is the presence of high content of nutritionally beneficial PUFA (polyunsaturated fatty acids), e.g. linoleic acid (18:2), linolenic (18:3) acids, which renders vegetable oil unstable for cooking. To overcome this problem, the vegetable oils are being hydrogenated to reduce the unsaturation and increase the saturation level. But as a consequence of the process, trans fatty acids are produced leading to rise in blood low-density lipoprotein (LDL) level, causing severe health-related issues in humans. This phenomenon led to creation of improved seed oils with reduced PUFA (Napier 2007). Studies have shown that 3-fatty acid desaturase (FAD3) helps in the conversion of linoleate (18:2) to α-linolenate (18:3) in membrane glycolipids (Hamada et al. 1994). However, RNAi has evolved as a precious tool for controlling this conversion event (Tomita et al. 2004; Hirai et al. 2007).

10.6.3.1 In Soybean

Linoleic acids (18:2) are converted to linolenic acids (18:3), by FAD3 enzymes during seed development in soybean (Bilyeu et al. 2003). Improved soybean oil quality requires reduction of PUFAs by downregulation of all three active members of FAD3 gene family (Bilyeu et al. 2006).

To achieve the silencing of these three active members of FAD3 family, researchers have employed a siRNA-producing transgene approach (Flores et al. 2008a). The highly conserved 318 bp region was amplified and cloned in RNAi cassette of pMU103 vector. The plant transformation was carried out with the pMUFAD vector. Seed-specific soybean glycinin gene promoter was used to drive expression of the RNAi cassette. The Northern blot analysis of mid-mature seeds (T1) for each of 10 RNAi lines revealed that in 5 out of 10 lines, no FAD3 transcripts were detected. The analysis of fatty acid profile of 23 RNAi lines revealed that 11 out of 23 transgenic lines contained reduced in α-linolenic acid (18:3). Contrastingly, these 11 transgenic lines also showed increase in linoleic acid (18:2) content.

This was the first successful attempt on developing a stably engineered soybean plant with controlled fatty acid profile achieved through RNAi-based technology.

The exchange of oleic acid to linoleic acid is catalyzed by FAD2 enzyme (Wang et al. 2015). *fad2–2* mutant soybean was generated using CRISPR-Cas9 system (Al Amin et al. 2019). Guide RNA targeting the *FAD2–2* loci in soybean was designed using various bioinformatics tools. *Agrobacterium tumefaciens*-based DNA transfer was used to successfully transform the binary vector (pCas9-AtU6-sgRNA) into soybean cotyledon. The Near-infrared spectroscopy also validated that transgenic seeds had an increase in oleic acid content up to (˜65.58%) than control. Indeed, in the same transgenic lines linoleic acid level decreased (˜16.08%). This is how CRISPR-Cas9 system can help us in manipulating the expression of *FAD2–2* gene which at the same time leads to reduction in linoleic acid content in soybean oil.

10.6.3.2 Cotton

The main components of seed oil of cotton (*Gossypium hirsutum*) are 15% oleic acid, 58% linoleic acid, and 26% palmitic acid. The presence of palmitic acid at a relatively high-level imparts stability to cotton oil, as a result of which they are appropriate for high-temperature frying applications, but is nutritionally undesirable, as this saturated fatty acid has a tendency of raising the level of blood LDL cholesterol. Therefore, there is an urgent requirement for oils with lower palmitic acid content but enriched in either oleic acid or stearic acid. Genetic modification of fatty acid synthesis is the only way to achieve such fatty acid profiles in several oilseed species. The activity of desaturase enzyme controlling the synthesis of major oil fatty acids can be downregulated by PTGS.

About 40% increase in rapeseed oil was noticed upon silencing of stearoyl-ACP _9-desaturase gene activity (Knutzon et al. 1992). However, in cotton seed this antisense and co-suppression technique both have some difficulties. Hence, to circumvent this limitation, research groups have employed hairpin RNA-mediated gene silencing strategy to reduce the expression of two key fatty acid desaturase genes, *ghSAD-1* and *ghFAD2-1* encoding stearoyl-acyl-carrier protein _9-desaturase and oleoyl-phosphatidylcholine _6-desaturase, respectively, in seeds. The hairpin RNA encoding constructs were designed against *ghSAD-1* or *ghFAD2-1* gene, followed by transformation in cotton. Significantly better stearic acid level was observed in the transgenic cotton seeds upon silencing of *ghSAD-1* gene. However, the downregulation of the *ghFAD2-1* gene resulted in increased oleic acid content compared to wild-type plants. Interestingly, in both high-stearic and high-oleic transgenic cotton lines, palmitic acid was significantly lowered. This is how RNAi can contribute to impart stability in seed oil of cotton without compromising its nutritional advantage.

10.6.4 RNAi in Hypoallergenic Plant

Many of the crops consumed daily as food creates problems. For example, consumption of legumes, tomato, potato, fruits like mango, apple, or even staple foods like rice, maize, wheat can result in exaggerated immune reaction, triggered by potential allergens present in those crops. This is termed as food allergy. In industrialized countries, these food allergies are a serious health concern. Food allergens affect nearly 6% children and 3–4% adults of total United States population (Sicherer and Sampson 2009). As per the data from the European Federation of Allergy and Airways Diseases Patients' Association, near about 2 per 100 adults and 8 per 100 children in the EU suffer from food-induced allergic responses (Helm and Burks 2000). The consumption of nutritional crops with toxins or any kind of unprocessed food can be potentially harmful, even leading to development of food poisoning, e.g. the most common symptoms manifested in human in response to allergenic fruit consumption include swelling of the lips, itching, and the throat infection; in

some rare cases consequence can be even more severe, resulting in onset of anaphylactic reactions (Amlot et al. 1987). As the food allergy symptoms are quite life threatening, people often try to avoid the consumption of those crops. Indeed, from nutritional point of view, this seems to be undesirable. This necessitates the development of hypoallergenic fruits and crops having either eliminated or tolerable limit of allergens.

10.6.4.1 Types of Plant Allergens

According to scientist, the typical food-borne allergens present in plants can be classified into 4 major categories, i.e. prolamin, cupin, profilin, and Bet v 1 (Radauer and Breiteneder 2007).

10.6.4.2 Strategies to Develop Hypoallergenic Crops

Currently two choices are available for developing hypoallergenic crops. One is the screening of germplasm lines to check for the absence or reduced content of specific allergenic proteins (Koppelman et al. 2001; Joseph et al. 2006; Ramos et al. 2009). It helps to recognize cultivars having natural hypoallergenic variants of known allergens. Another way out is generation of transgenic plants in which metabolic pathways are engineered to achieve plants producing lower allergenic proteins (Dodo et al. 2008a; Herman et al. 2003; Chu et al. 2008).

PTGS contributes to development of transgenic hypoallergenic crops by targeting their metabolic pathway genes. PTGS strategies can be categorized into two mechanistically similar approaches, i.e. RNA interference (RNAi) (Chu et al. 2008; Dodo et al. 2008b) or related approaches such as co-suppression (Herman et al. 2003). In both cases PTGS is triggered by the generation of aberrant dsRNAs (Kusaba 2004; Hannon 2002).

Peanut

Allergy in response to peanut consumption is very frequent event for triggering food-induced anaphylaxis owing to the presence of allergen in peanut (Bock et al. 2007) and its prevalence is increasing (Burks 2003). Ara h 1, Ara h 2, and Ara h 3 are the potential allergens present in peanut seed (Kang et al. 2007). Ara h 2 which is a17.5 kDa glycoprotein is the most effective allergen among the three allergens (Burks et al. 1992; Burks et al. 1995; Sen et al. 2002). Indeed, its potency is nearly 50-fold greater than Ara h 1 (Koppelman et al. 2001; Koppelman et al. 2004). It is coded by two homologous genes, *Ara h 2.01* and *Ara h 2.02* (Ramos et al. 2006). Several groups have tried to silence the *Ara h 2* gene in peanut using RNAi approach.

The first attempt toward the development of hypoallergenic peanut was done by creating RNAi against *Ara h 2*gene (Dodo et al. 2008a).

The western blotting and ELISA experiments confirmed that the crude extract of T0 peanut plants were devoid of Ara h 2 protein. The ELISA performed on patients also confirmed that the allergenic effectiveness of the transgenic peanut was significantly reduced compared with wild type.

Tomato

Tomato (*Lycopersicon esculentum*) is a worldwide consumed crop because of its high content of lycopene, beta-carotene, and other extremely beneficial components (Giuliano et al. 1993; Crozier et al. 1997; Fraser and Bramley 2004). But consumption of tomato in diet often leads to onset of severe symptoms in the body due to the presence of a number of proteins with very high allergenic potential. Lyc e 2 and Lyc e 3 (Le et al. 2006a, b) are the two major allergens of tomato (Andersen and Løwenstein 1978; Eriksson 1978; Ebner et al. 1996).

Lyc e 1 belongs to profilin family of plant allergens (Westphal et al. 2004). Lyc e 1 triggers the release of inflammatory mediators from human basophils (Westphal et al. 2004). High degree of identity at the nucleotide level (88.1% identity) between the two isoforms of Lyc e 1-Lyc e 1.01 and Lyc e 1.02, allows the construction of only single RNAi construct that can silence both isoforms. The cDNA fragment of Lyc e 1.02 in sense and antisense position was cloned in plant transformation vector pK7GWIWG2(II) followed by transformation into tomato. The transgenic plant showed about 10-fold reduction in accumulation of Lyc e 1 as revealed by ELISA (Le et al. 2006a).

Lyc e 3, present in tomato is characterized as IgE-reactive polypeptide with mol. wt of 9-kDa, and recognized as a non-specific lipid transfer protein (van Ree et al. 2000). The pBin-LTPG1_RNAi and pBin-LTPG2_RNAi constructs were generated by using the pUC-RNAi vector to clone the entire coding region of *LTPG1* or *LTPG2* in sense and antisense orientation. Two different sets of transgenic plants were generated with the two different RNAi constructs. The results revealed significant reduction of Lyc c 3 level in fruits of transgenic plants compared to wild-type plants (Le et al. 2006b).

Apple

Apple is an important fruit crop which belongs to Rosaceae family and the subfamily Maloideae. Majority of the population consume apple in their healthy diet, however, the intake of apple leads to the development of allergy symptoms in few individuals. Indeed, it was also noticed that 70% individuals those who are allergic to birch pollen are also allergic to apple (Dreborg and Foucard 1983). The IgE antibodies, specific to pollen, cross-react with highly homologous proteins present in fruit and vegetables, causing allergic symptoms (Andersen and Løwenstein 1978; Eriksson 1978; Ebner et al. 1996). Research studies have shown that Mal d 1 protein present in apple bears 64.5% homology to birch pollen allergen.

Studies revealed that *Mal d 1* gene family representative contains either a single intron or is intronless (Gao et al. 2005). Those intron-containing Mal d 1 genes were targeted by researches through RNAi construct. Studies have indicated that among the several isoforms of *Mal d 1* gene, *Mal d 1b* is the most abundant, not only at the transcriptional level but also at the protein level. Moreover, it is also significantly expressed in ripe fruit (Helsper et al. 2002; Puehringer et al. 2003). Therefore, Mal d 1 gene isoform was considered as best candidate for silencing.

Gilissen and his group (Gilissen et al. 2005b) first isolated genomic DNA from Apple cultivar gala and amplified these regions resulting in hairpin RNA construct. The amplified fragment was further cloned in binary vector pBINPLUS23 followed by Agrobacterium-mediated transformation in Elstar cultivar. PCR analysis demonstrated the presence of the construct in six out of nine selected plantlets. Moreover, to judge the allergenicity in these plantlets, SPT analysis was carried out in three apple-allergic patients. The results indicated that the allergenicity of wild-type plantlets was significantly higher than five of the transformants. Immunoblotting confirmed at least 10-fold reduction of *Mal d 1* expression in the transformants. Hence, with this approach it became feasible to develop apple with reduced allergenicity.

Cassava

The most important and economical root crop in the world is Cassava (*Manihot esculenta* Crantz). Mostly, in the tropical and subtropical regions of the world, about 500 million people consume cassava as a staple food. However, the consumption of unprocessed cassava leads to acute human toxicity or permanent disability due to presence of plant toxins.

Cassava has a high content of the linamarin and lotaustralin, potent toxic cyanogenic glucosides in all of its tissues except seed (Conn 1994). Moreover, partial processing may result in high cyanide exposure leading to permanent paralysis of the legs, while chronic exposure to these cyanides gives rise to symptoms like hyperthyroidism and neurological disorders, including tropical ataxic neuropathy (Mlingi et al. 1992).

So, there is a need of developing acyanogenic cassava to make this staple crop safe and accessible. The two cytochromes—P450 CYP79D1 and CYP79D2, catalyze the biosynthesis of the cyanogenic glucosides—linamarin and lotaustralin in cassava (Jørgensen et al. 2011). Researchers have developed the antisense CYP79D1 and CYP79D2 constructs under the control of leaf-specific, Cab1 promoters and utilized *Agrobacterium*-mediated T-DNA transformation to introduce the 5'ends (650 bp) of those two genes (Siritunga and Sayre 2003). This results in selective reduction of *CYP79D1* and *CYP79D2* gene expression in either root or leaves. In transformants with altered *CYP79D1* and *CYP79D2* transcripts, leaf linamarin content showed a reduction, ranging between 94% and 60%, respectively, compared to WT. Therefore, this RNAi approach leads to a model for developing marketable acyanogenic cassava as an alternative food source.

Tobacco

In cultivated tobacco, during the curing period, nicotine present in the senescing leaves undergoes an enzymatically catalyzed N-demethylation and converts to nornicotine, a secondary alkaloid (Hao and Yeoman 1998; Chelvarajan et al. 1993). As this nornicotine can stimulate the aberrant glycation of proteins and modify the pharmacological nature of prednisone and perhaps other commonly used steroid drugs, consuming tobacco often results in unwanted health-associated effects in smokers (Dickerson and Janda 2002). However, inhibiting the activity of nicotine demethylase could be an effective approach for reducing the level of distinct carcinogens present in tobacco products. Researchers have developed an optimized RNAi construct (82E4Ri298) for silencing the *N*-demethylase (CYP82E4) gene and thereby reducing the conversion of nicotine to nornicotine in tobacco (*Nicotiana tabacum*). The results indicate the effectiveness of RNAi in developing transgenic tobacco lines with reduced nornicotine content. Another group of scientists have developed transgenic burley-tobacco breeding line, DH98-325-5, with RNAi-silenced nicotine demethylase, where they were able to find six-fold decrease in nornicotine content (Lewis et al. 2008).

10.6.5 Reduction of Alkaloid in Crops by RNAi

Alkaloids are mostly basic nitrogen containing naturally occurring compounds. People consume alkaloids present in crops as their regular part of the diet. Pyrrolizidine alkaloids are the main cause of concern due to their bioactivation of reactive alkylating intermediates. Quinolizidine alkaloids, β-carboline alkaloids, ergot alkaloids, and steroid alkaloids are mostly neurotoxins and are active without bioactivation (Koleva et al. 2012). Thus, being aware of the risk, regulatory agencies have taken suitable measures for most alkaloids. The risk is coming from the alkaloids known to be present in the modern food chain piperine, nicotine, theobromine, and theophylline. Therefore, RNAi can be the possible strategy to reduce the alkaloid content in the crops, to make it more safer for intake.

10.6.5.1 Strategies for Reduction of Nicotine in Leaves of Tobacco

The motivation always exists for tobacco cultivars to develop tobacco with lower nicotine level for the purpose of facilitating fulfillment with expected tobacco product. There are mandates for lowering of nicotine level or reduction of carcinogens derived from tobacco-specific nitrosamines (TSNA). Among many plant secondary metabolites, pyridine is the most studied alkaloid from tobacco. Nicotine constitutes greater than 90% of the total alkaloid content in most tobacco genotypes. (Sisson and Severson 2016) Nicotine has an implicated role as a precursor to one of the tobacco-specific TSNAs, a potential carcinogen (Hecht 1998, 2003).

RNAi as Tool to Reduce Nicotine Content

Berberine bridge enzyme-like (BBL) gene family is known to be responsible for alkaloid formation. The BBL is flavin-containing oxidase enzyme and acts in the final step of nicotine production (Kajikawa et al. 2011). In laboratory and greenhouse system, the suppression of BBL gene family led to reduced nicotine phenotype (Kajikawa et al. 2011). BBLa, BBLb, BBLc, BBLd are the different isoforms of *BBL* gene (Kajikawa et al. 2011). To enable the silencing of the gene, a 212 bp conserved region was selected to maximize the probability of suppression. This fragment from BBLa is 94, 93, and 84% identical to the analogous region of BBLb, BBLc, BBLd, respectively. Thus anti-BBL RNAi was generated especially against BBLa sequence. This RNAi-based antisense construct was transformed into tobacco via Agrobacterium-mediated transformation. Six among the ten tested RNAi lines exhibited significantly reduced nicotine level than that observed in the untransformed control line k326.

10.7 Conclusions

Conventional breeding techniques, due to long breeding cycles of crops as well as the potentially random effects of traditional breeding, are incapable to meet the market demand to address global emerging food crisis. On the other hand, genetic engineering approach has got limited success because of its inability to silence the target genes in a tissue-specific manner to increase the metabolic flux toward the desired product. In this scenario, RNAi technique has got an edge to improve the nutritional quality of crops through gene silencing with precision, in a specific tissue of the plant. In more recent advancement of metabolic engineering, CRISPR/Cas9 has emerged as a more sophisticated tool, where multiple genes can be targeted to activate or repress a metabolic pathway. A set of scaffold RNAs can be mediated by CRISPR/Cas9 to manipulate an entire metabolic pathway, which is governed by many proteins/enzymes and guided by different transcriptional controls (activating or repressing) at various steps (Zalatan et al. 2015). Thereby, it has emerged as an effective tool to operate the pathways in a manner that enables concurrent regulation of independent gene targets.

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Chapter 11
Gene and Genome Editing with CRISPR/Cas Systems for Fruit and Vegetable Improvement

Semih Arbatli, Julia Weiss, and Marcos Egea-Cortines

Abstract Ever since the advent of agriculture, breeding new varieties has relied upon crosses between individuals from a single species, and since the early twentieth century with relatives or via mutagenesis. Two major problems have been found time and again. First, combining genomes to improve a character often times causes decreases in other traits as a result of genetic linkage. The second is that natural variation does not always comprise all the possibilities a genome may have in terms of allelic combinations suitable for further improving a set of characters. In the last twenty years a number of technologies have been developed allowing the perturbation of a single gene. Development of genome editing technologies includes zinc finger nucleases (ZFNs), transcription activator-like effector nucleases (TALENs) and Clustered Regularly Interspaced Palindromic Repeats (CRISPR). Here we review current methodologies regarding to the use of gRNA targeted gene and genome editing strategies by various CRISPR/Cas9 systems in agriculture. The molecular mechanism of DNA modification by CRISPR/Cas relies on guide RNA molecules comprising 20–25 DNA bases homologous to the target locus. This has opened the possibility of tackling single loci or multiple paralogs in a gene family. Importantly, complex genomes with polyploid structures such as wheat or camelina have been successfully engineered with single guides. This opens a new window of opportunities to engineer gene families, pathways and complex genomes that was unfeasible before the advent of CRISPR/Cas.

Keyword Genome editing · CRISPR/Cas · Multilocus edition

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G. Tang et al. (eds.), *RNA-Based Technologies for Functional Genomics in Plants*, Concepts and Strategies in Plant Sciences, https://doi.org/10.1007/978-3-030-64994-4_11

11.1 From Chromosome Transfer to Single Gene Transfer

Plant breeding refers to the cross-fertilization of two parents in order to obtain desired traits. Throughout the history, humans have modified nearly all plant species of economic interest in order to enhance quality and increase the yield obtained.

At the end of the nineteenth century, the scientific plant breeding studies emerged in order to achieve new and improved genetic variations of the commonly used crop species in all aspects of cultivation (Hallauer 2007). Later on, in the twentieth century, the advancement of plant breeding started to ascend with the formation of agricultural research centers and development of private companies. New and faster methods have been expanded to match raised requirements to agricultural needs. The crosses with wild relatives had a crucial role in the development of the current diversity of cultivars. The introgression of genes for breeding purposes via interspecific hybridization from non-cultivated plants species to a related crop species is an additional method where natural variation was exploited (Goodman et al. 1987).

To illustrate, an early example of interspecific hybridization to a cultivated crop species is wheat. Gene transfer of wheat with the aforementioned method has been achieved in 1930 with the transfer of resistance genes from *Triticum tauschii* into *T. aestivum* (reviewed in [Hoisington et al. 1999]). In 1936, a resistance gene was transferred from *Solanum pimpinellifolium* to the cultivated tomato (*S. esculentum*) (reviewed in [Goodman et al. 1987]). Crop improvement continued with interspecific gene transfer, and in those cases where it was feasible via untargeted mutagenesis (Menda et al. 2004; Sikora et al. 2011). The random mutagenesis using chemicals or ionic radiations has given rise to "Targeting Induced Local Lesions in Genomes" (TILLING) (Comai and Henikoff 2006). There are successful examples of TILLING on various plant species such as *Solanum tuberosum*, *Cucumis melo* and *Cucumis sativus* (Elias et al. 2009; González et al. 2011; Boualem et al. 2014). While TILLING has many advantages, such as obtaining non-transgenic material, an extensive genetic work has to be performed to isolate the single mutant and introduce it in elite germplasm.

In the 1980s, the age of plant biotechnology started. *Agrobacterium tumefaciens* which is a gram-negative, plant pathogenic soil bacterium, made it possible to develop horizontal gene transfer technologies, thus opening a new era for breeding based on different conceptual principles (Chilton et al. 1977; Herrera-Estrella et al. 1983). Thereafter, different methods of gene transfer have been developed such as microinjection process and particle bombardment. These methods are relying on the injection of desired DNA into the target plant or bombarding the plant with tiny particles that contain the gene of interest, thus achieving single gene transfer in plants that are not natural hosts of *Agrobacterium.* But the rationale behind these technologies was to engineer plant genomes one gene at a time, in sharp contrast to the classic breeding programs where one or several genes encompassing chromosome fragments, are

crossed into a line and then it is backcrossed for at least eight generations to clean the original genome from unwanted fragments.

Genetically modified (GM) crops are agricultural crops that express a certain gene or genes inserted in the host genome, that do not possess it naturally. They have been arguably the biggest success in terms of technology development and they amount for over 50% of the total crop area in the World (FAO). They created a large debate based on the presence of resistance genes used during the transformation process as they were thought as a threat to human health if they would be transferred to the human gut microbiome. In a recent study conducted on the cultivated sweet potato clone “Huachano,” the presence of an additional transfer DNA (T-DNA) has been detected in 291 tested accessions of cultivated sweet potato (Kyndt et al. 2015). This research also displays the presence of a specific T-DNA among all cultivated sweet potato species, excluding the wild relatives. Hence, the study points out the possibility of an *Agrobacterium* infection on sweet potato in history. It has been suggested that this T-DNA transfer provided a benefit in an agricultural manner, thus made it preferable through its domestication process (Kyndt et al. 2015). A recent work examined 275 dicot species and found presence of T-DNA in 23 species such as *Arachis*, *Nissolia*, *Camellia* or *Dianthus* (Matveeva and Otten 2019). This new data shows that the horizontal gene transfer by *Agrobacterium* is rather common in nature. However, transgenic systems relying on random insertions in the genome such as those based on *Agrobacterium* or biolistic, do show high variability in terms of transgene stability, level of expression and copy number.

11.2 Gene Targeting

Gene targeting relies on Homologous Recombination (HR) whereby a gene sequence is replaced by a nearly identical sequence albeit mutated (Reiss 2003). The HR-based DNA repair is a DNA protection pathway against structural damages involving double-strand DNA breaks. Naturally there are different ways of foreign DNA integration to the original genome. These are improper recombination, Non-Homologous End Joining (NHEJ) and Single Strand Annealing. However, methods relying on HR are limited from certain aspects such as difficult screening strategies, time-consuming experimental setup and potential mutagenic effects due to the low target efficiency and improper binding (Gaj et al. 2013). While homologous recombination worked very well in several genetic models such as yeast or mice, it was not amenable in others where the genetic tools were highly developed such as Drosophila or plants. The only exception is *Physcomitrella patens,* a haploid moss, where homologous recombination was rapidly achieved (Schaefer and Zrÿd 1997).

11.3 First-Generation Genome Editing Technologies

One standard tool in molecular biology labs is DNA digestion with restriction enzymes. They recognize short sequences, mostly palindromic between four and twelve bases long. This made them unsuitable to target one locus as a standard enzyme may digest a single genome into hundreds to hundreds of thousands of fragments. Thus, early efforts were done to obtain engineered nucleases ideally recognizing a single target DNA stretch in a genome. Considering DNA as a random molecule comprising four bases, which is a very gross scientific misconception, one can argue that a DNA fragment of roughly 15–20 bases should suffice to identify a single fragment in the genome just randomly, i.e. the probability of finding precisely 15 bases is $(1/4)^{15}$. This means that obtaining nucleases with specificities of 18–25 bases should give enough target specificity to tackle a single locus for genome engineering.

The use of engineered nucleases (ENs) was first applied in 1981 by Wallace et al. (Bruce Wallace et al. 1981). Since then, four different types of defined nuclease classes have been developed: Zinc Finger Nucleases (ZFNs), Meganucleases, Transcription Activator-Like Effector Nucleases (TALENs) and Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR)-based technologies (Metje-Sprink et al. 2019). To date, ZFNs, TALENs and recently CRISPR/Cas9 systems have been commonly used in the field of plant genetics.

The first-generation genome editing technologies rely on a sequence-specific DNA-binding domain and a non-specific nuclease domain. The system relies on the use of site-specific nucleases (SSNs). SSNs are probably the most well-known method in the field of gene editing (Sivanandhan et al. 2016). SSNs cleave the target DNA fragment, thus creating Double-Strand Break (DSB) (Sanagala et al. 2017). These DSBs can be repaired through NHEJ and Homology directed recombination (HDR) pathways, consequently creating insertions or deletions (INDELs) on the targeted region. Gene targeting strategies with DSBs administer an exogenous template for the naturally occurring repair mechanism (Carroll 2011).

NHEJ pathway has been commonly preferred over HDR due to low efficiency rates of conducted studies using HDR (Schindele et al. 2018). ZFN and TALEN-based systems have been applied as a first-generation genome editing systems followed by the CRISPR/Cas-based gene modification approaches due to several aspects explained later on throughout the chapter (Fig. 11.1).

11.4 Zinc Finger Nuclease Genome Editing

ZFNs are a class of nucleases that consist of separate DNA-cleavage and DNA-binding domains (Carroll 2011). Zinc finger (ZF) domains that are attached to the FokI domain recognize the target sequence. *FokI* is a natural type-IIS restriction enzyme, with a non-specific target sequence (Kim and Chandrasegaran 1994). The identification of alterable properties on the *FokI* domain introduced the possibilities of DNA

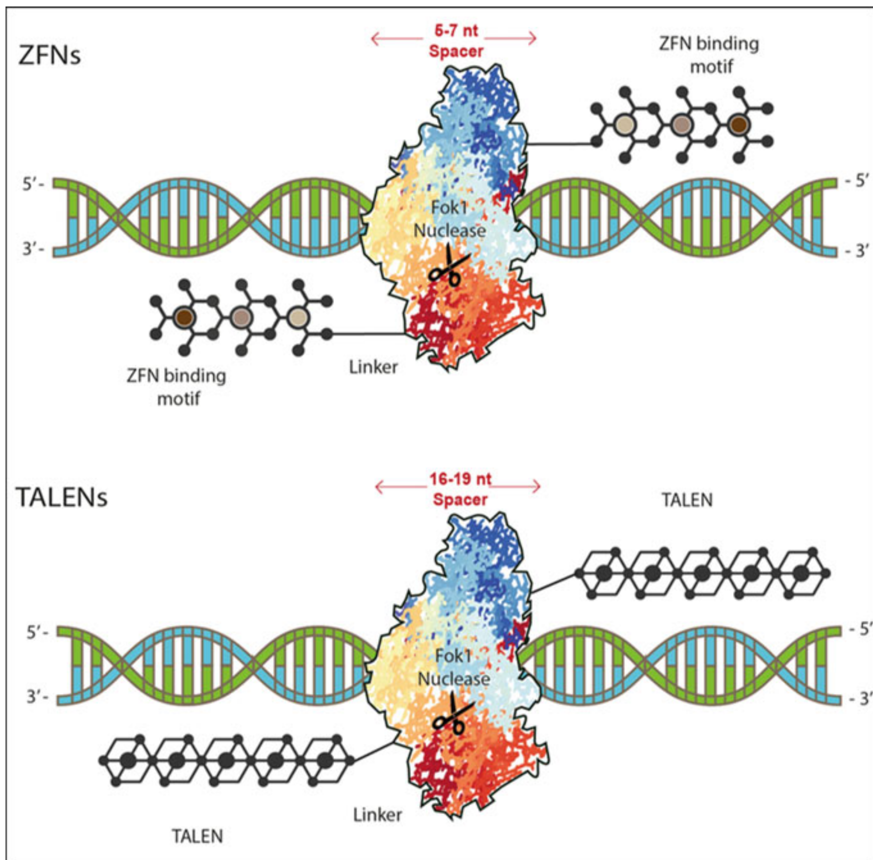

Fig. 11.1 Schematic representation of ZFN and TALEN systems on target DNA fragment resulting with DSBs and creation of INDELS (Fok1 nuclease structure obtained from Protein Database (PDB), [Wah et al. 1997])

cleavage in a site-specific manner, without the necessity of previously engineered sites for the target to bind and function (Sanagala et al. 2017). The advancement of ZFN-mediated gene targeting enables the modification on plant genome specifically by homology directed repair (HDR) of target DSB (Malzahn et al. 2017). ZFNs are able to recognize 18–24 bp of DNA sequences with a 5–7 bp spacer on both ends (Pabo et al. 2001). Binding of ZFN to the target site pursues with the production of DSBs with the Fok1 cleavage domain.

First gene editing reports using ZFN system in plants were published in 2005 by Lloyd and his colleagues (Lloyd et al. 2005). The study conducted with Arabidopsis as a model organism and the ZFN-induced mutations has been characterized showing that the NHEJ-based methods are more likely to be effective in comparison to the HR-based mutagenesis.

11.5 TALEN-Based Genome Editing

The modification of the transcriptional activator-like effector (TALE) domains to achieve successful gene editing strategy resulted in the development of TALENs, that are based on a TALE domain fused with *FokI* nuclease site. TALENs have the capability to recognize 18–20 bp long stretches on the target region. TAL effectors belong to *Xanthonomas sp.* The mechanism simply uses type III secretion system (T3SSs) for the translocation process into the cells (White et al. 2009). T3SSs are bacterial structures providing gram-negative pathogens with the ability to inject effector proteins into host cell cytoplasm. Some of the TAL effectors have been known for their transcriptional activation on target region (Sugio et al. 2007; Li et al. 2011). A repetitive region containing various number of amino acids (~34 bp nuclear localization motif) along with the transcriptional activation domain are the building blocks of the TAL structure (Gürlebeck et al. 2006). These repetitive regions act as the target recognition agents (Moscou and Bogdanove 2009). As they are universally recognized by the cellular machinery, they can be used for gene alteration as a gene modification tool in plants (Boch et al. 2009).

There are different TALE variations with different binding specificities aiming to increase the target specificity (Sprink et al. 2015). For instance, Hax3, which can recognize a target sequence of 12 bp long, has been used for the construction of a genetically engineered nuclease for targeted mutagenesis on *Nicotiana benthamiana* plants (Mahfouz et al. 2011). Since then, TALEN-mediated genome engineering studies have been applied to various plant species (reviewed in [Sanagala et al. 2017]).

Custom engineering of ZFN proteins is a time-consuming process with a low success rate. This hampers the widespread use of the ZFN system and led to the development of new efficient and precise gene editing techniques. (Joung et al. 2010). The problem of low success rate is directly related to the affinity of the particular ZFN. To increase the specificity, each ZFN construct has been designed with at least 3 fingers. However, the contribution of each finger does not occur at the same level, compared to each other. Moreover, at a certain point, the amount of added fingers might also reduce the target binding affinity due to various factors such as complex chromatin structure (Carroll 2011). Currently, ZFNs are the least preferred gene modification tool due to limited number of target-cleaving sites and high number of off-target cleaves leading to the low target specificity and restricted experimental systems.

TALENs are another system that is also based on the TAL gene responsible from the manipulation of host gene expression (Li et al. 2011). TALENs are functional in order to manipulate eukaryotic genomes in the manners of target identification and cleavage. However, there are limited number of modified plant species based on TALENs (Arabidopsis, barley, tobacco, rice, *Brachypodium*, tomato, maize, *Nicotiana bentamiana*, soybean, sugarcane, potato and wheat) (reviewed in [Sanagala et al. 2017; Malzahn et al. 2017]). Another issue regarding to the insufficiency of TALENs as a gene modification tool is its time-consuming procedures as long as

complex and labor-intensive experimental setup. These crucial problems are main inadequate aspects of TALEN-based gene alteration systems.

11.6 The CRISPR/Cas Technologies

CRISPR was first described in *E.coli* by Ishino et al. in 1987 (Ishino et al. 1987). Naturally, CRISPR system is an adaptive defense response of archaea and bacteria to prevent viral invasions (Bhaya et al. 2011). The discovery of CRISPR/Cas9 systems was an important breakthrough in the development of precise gene editing studies. CRISPR-based systems are having a different origin than other gene alteration systems and they are common in various species. The first success achieved by CRISPR/Cas system was in mammalian cells (Jinek et al. 2012). The first plant gene editing study using CRISPR/Cas systems has been published by Feng et al. in 2013 (Feng et al. 2013). This achievement has been followed by its immediate implementation on various plant species such as Arabidopsis, rice, wheat and tobacco (Feng et al. 2013; Upadhyay et al. 2013; Jiang et al. 2013). Later on, different Cas variants isolated from different species such as *Streptococcus thermophilus* have been developed for further gene alteration processes (Steinert et al. 2015). Studies continued with successfully edited CRISPR/Cas plants including but not limited to barley, cotton, dandelion, flax, liverwort, soybean, sorghum, tomato or petunia, to name a few (Malzahn et al. 2017).

The CRISPR/Cas systems ease the whole gene alteration processes and proved itself as a successful gene modification tool in the field of plant genetics, in comparison to the first-generation genome editing techniques based on TALEN or Zinc Finger Nucleases. Cas9-induced mutagenesis has been used to target cis-regulatory-elements (CREs) of quantitative traits (Rodríguez-Leal et al. 2017).

CRISPR system involves two different classes of RNA-guided nuclease effectors. Class 1 effectors are related to the utilization of multi-protein complexes, while Class 2 effectors act as unique agents, single component effector proteins (Zetsche et al. 2015). The Class 1 effectors are consisting of type I, type III and type IV systems while Class 2 effectors are containing type II, type V and type VI (Makarova et al. 2015). Especially the effector modules are distinctive among the various types of CRISPR/Cas systems (Charpentier et al. 2015). The Class 1 effectors are able to form an effector complex including CRISPR RNA (crRNA) and certain Cas proteins, while Class 2 effectors use a large Cas module associated with crRNAs to obtain target specificity. crRNA is the transcribed RNA in the presence of a secondary viral attack after the incorporation of a spacer sequence after the primary intrusion. Each transcribed crRNA is carrying both nucleotide repeats and spacer. Another product of the aforementioned formation is the trans-activating crRNA (tracrRNA) which is another molecule that binds to Cas structure in order to lead it to the target site.

11.7 Types of CRISPR/Cas Systems

11.7.1 CRISPR/Cas9

CRISPR/Cas9 is the best characterized type among all CRISPR/Cas precise gene modification tools. The ortholog of Cas9, derived from *S. pyrogenes* (spCas9), is commonly used in the field of plant genetics. Moreover, another ortholog derived from *Staphylococcus aureus* (saCas9) has been proven to have similar success in order to modify plant genomes, suggesting a second alternative for the common spCas9-based plant gene modification studies (Steinert et al. 2015).

The CRISPR/Cas9 system basically works with a designed, short synthetic guide RNA (gRNA) fragment (~20 bp) responsible for target identification and binding and a nuclease with a capacity to cleave 3–4 bases after the so-called protospacer adjacent motif (PAM) (Jinek et al. 2012). These PAM regions are consisting of the sequence 5' NGG. The Cas nucleases are generally a composition of HNH domain and a RuvC-like domain (Jaganathan et al. 2018). The first system based on the spCas9 was developed by Jinek et al. Later on, various orthologs of Cas9 have been identified and are available for further plant gene editing studies (Fig. 11.2).

11.7.2 Crispr/CAS12a (Cpf1)

CRISPR/CAS12a (formerly known as Cpf1) is a Class 2 type V gene modification system that diverges from the Cas9-based systems in several ways. First of all, target specificity of the CRISPR/Cas12a system requires a minimum of 22 nucleotides (Lei et al. 2017). It is a longer region compared to the traditional CRISPR/Cas9-based gene editing systems and increases the target binding efficiency while reducing the off-target effects (Chen et al. 2018).

Moreover, the Cas12a-based system requires Thymine-rich PAM sequences (5'-TTTV-3') while Cas9 requires Guanidine-rich PAM structures (5'-NGG-3'), therefore increasing the amount of possible approaches by offering a higher number of potential target regions. It is further characterized by the creation of staggered DSB ends distal from the PAM region instead of the blunt-end DSBs obtained by the traditional Cas9 (SpCas9) system (reviewed in [Schindele et al. 2018]). The cleavage of the target strand occurs after the 23rd nucleotide while non-target strand cleavages occur after the 18th nucleotide, producing a region consisting of a 5-nucleotide overhang on the 5' end. SpCas9 requires a gRNA with approximately 100 nucleotides, while CRISPR-Cas12a system requires a crRNA of 43 nucleotides in length (Zetsche et al. 2015).

A variation of Cas12a nuclease isolated from *Leptotrichia shahii* is presenting dual nuclease activity, therefore capable of targeting single-stranded RNA (Zaidi et al. 2017). The first Cas12a-based experiments in plant genetics have been conducted on tobacco and rice (Endo et al. 2016; Xu et al. 2017). Since then, three different

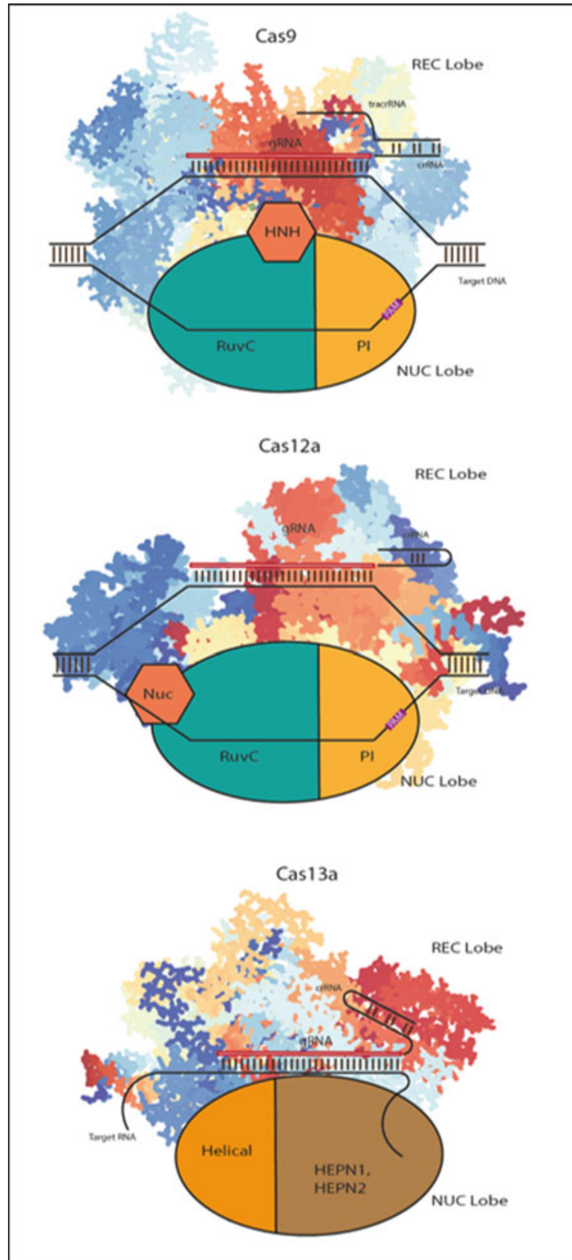

Fig. 11.2 Basic representation of Cas9, Cas12a and Cas13a system (Representative background models for Cas9, Cas12a, Cas13 belong to spCas9, FnCas12a and LbuCas13a, respectively) (Nishimasu et al. 2014; Swarts and Jinek 2019; Liu et al. 2017)

variants of Cas12a have been introduced into various model plant species. These are including but not limited to *Acidaminococcus sp.* Cas12a (AsCas12a), *Francisella novicida* Cas12a (FnCas12a) and *Lachnospiracea bacterium* ND2006 (LbCas12a) (reviewed in [Jia et al., n.d.]).

11.7.3 Crispr/Cas 13(C2c2)

Both Cas9 and the Cas12 have been commonly used in order to create ssDNA breaks in plant DNA. However, CRISPR/Cas-based alterations of single-stranded RNA (ssRNA) fragments have been achieved only with the development of CRISPR/Cas13 (previously called C2c2) systems, entitled as a "new swiss army knife" for plant biologists (reviewed in [Wolter and Puchta 2018]). In 2015, Shmakov et al. identified 3 novel Class 2 effectors named C2c1, C2c2 and C2c3 (Shmakov et al. 2015). Both C2c1 and C2c3 represent characteristics similar to Cas12. However, C2c2 diverges from other Class 2 effectors due to its distinctive features. Therefore, the system was later on classified as a new type of effectors. CRISPR/Cas13 is a Class 2, type VI ribonuclease gene modification system which confers immunity against phage invasions (Schindele et al. 2018).

The Cas13 system comprises a diverse structure with nuclease and recognition domains different from other Class 2 effector systems. Similar to Cas12, Cas13 systems also do not require tracrRNA to process pre-crRNA (reviewed in [Liu et al. 2017]). In Cas13, target RNA cleavage activity and the crRNA maturation process are distinct from each other (reviewed in [Wolter and Puchta 2018]).

Another structural difference compared to Cas9 and Cas12 consists in the position of higher eukaryotes and prokaryotes nucleotide binding domain (HEPN) located on the outer surface. These domains are enabling the protein to cleave target RNA which is positioned on the outside of the binding region. However, this ability also causes the possible non-specific cleavage of any other RNAs that are present in that area (East-Seletsky et al. 2016).

11.7.4 Using Crispr to Modify Single Genes

Dicot plants have been the main objects of gene editing by CRISPR/Cas systems, as they are comprising most of the agricultural plants. Initial experiments were performed by Feng et al. in 2013 (Feng et al. 2013). These studies targeted three different genes of the Arabidopsis genome, *Brassinosteroid insensitive1 (BRRI1), Jasmonate-zim-domain protein 1 (JAZ1)* and *Gibberellic acid insensitive (GAI),* and a significant efficiency (26–84%) of mutagenesis was observed. Since then, many applications of the CRISPR/Cas systems targeting the Arabidopsis genome were reported (Miki et al. 2018).

Tomato (*Solanum lycopersicum L.*) is an important crop model for fruit quality improvement, and the genome structure is well characterized (Pan et al. 2016). Successful CRISPR/Cas9-based interference of the tomato gene *SlAGO7* (*ARGONAUTE*) demonstrated the possibility of precise gene editing in tomato (Brooks et al. 2014). Another study targeting the *SHR* (*SHORT-ROOT*) gene, which regulates *SCARECROW* (*SCR*) transcription factor gene expression, using the CRISPR/Cas system was performed by Ron et al. and results indicate a correlation between the tomato *SHR* gene and both root length and the *SCR* gene expression (Ron et al. 2014). Yet another experiment in tomato, conducted by Ueta et al., showed that the interruption of *SlAA9, a* gene related to parthenocarpy, resulted in the development of seedless fruits and alterations in the leaf morphology (Ueta et al. 2017).

Soybean (*Glycine sp.*) is an important crop containing a high protein content along with physiologically active substances in the seeds. The first CRISPR/Cas-mediated gene modification experiment on soybean was conducted by Cai et al., targeting two genomic sites on chromosome 4 and leading to small deletions and insertions in this region (Cai et al. 2015). Another approach was the interference of *Rj4* gene, which plays a role in nodulation inhibition in many strains of *Bradyrhizobium elkanii*, using the CRISPR/Cas9 system (Tang et al. 2016, p.4).

Cotton (*Gossypium sp.)* is another major crop in many areas. Its uses include but are not limited to fiber and biofuel production (reviewed in [Jaganathan et al. 2018]). The sequencing of the *Gossypium hirsutum* genome was published in 2015 (Li et al. 2015). First targeted gene editing experiments in cotton using CRISPR/Cas9 system have been accomplished by Janga et al. using transgenic cotton bearing an integration of the Green fluorescent protein (GFP) (Janga et al. 2017). The generated GFP regions were targeted by CRISPR/Cas9 and examined in order to identify the pathways related to the DSB utilization. Another study aimed to interfere with two genes of the cotton genome—*Cloroplastos alterados 1* (*GhCLA1*) and *Vacuolar H-pyrophosphatase* (*GhVP*)—and results demonstrated a high mutational efficiency (47.6–81.8%) (Chen et al. 2017).

Grape (*Vitis sp.*) is another plant of high economic importance. Five different CRISPR/Cas9 target sites within the *Vitis vinifera* genome (protospacer adjacent motif or PAM) were identified (TGG, AGG, GGG, CGG, NGG) and found to be uniformly distributed among the grape genome (Wang et al. 2016). The CRISPR/Cas9 system has been used to mediate interference of the *I-idonate dehydrogenase* (*IdnDH*) gene on "Chardonnay" suspension cells following regeneration of grape plantlets, showing the absence of off-target mutations (Ren et al. 2016). Nakajima and his colleagues targeted the *Phytoene desaturase* (*VvPDS*) gene which is related to the albino leaf formation with CRISPR/Cas-mediated mutagenesis (Nakajima et al. 2017). The study demonstrates that old leaves have a high mutation rate compared to the newly formed leaves, suggesting an increased incidence of DSBs or impaired repair mechanisms in the old leaf samples (reviewed in [Jaganathan et al. 2018]). Another study targeting the *MLO*-7, a gene related to an increased resistance to powdery mildew disease on grapevine protoplasts, resulted in the generation of resistant mutants (Malnoy et al. 2016).

Sweet orange (*Citrus sinensis*) is another commonly produced and consumed fruit providing 60% of the total citrus production worldwide (Xu et al. 2013). In a study conducted by Jia et al., sweet orange *Phytoene desaturase* gene (*CsPDS*) related to citrus canker disease resistance, has been targeted with a novel Xcc (*Xanthomonas citri subsp. citri*)-facilitated agroinfiltration. The study resulted in a successful mutagenesis of *CsPDS* genes (3.2–3.9% mutation rate) along with the absence of off-target effects (Jia and Wang 2014).

There are several monocot plant species where CRISPR/Cas gene modification systems have been successfully introduced (reviewed in [Jaganathan et al. 2018]). In a study conducted on barley genome, an interference on the *Endo-N-acetyl-b-D-glucosaminidase* gene (*ENGase*) has been mediated while using both *Agrobacterium*-mediated transformation and particle bombardment technique (Kapusi et al. 2017). The study states that among all observed T0 and T1 mutant barley lines, 78% showed mutational efficiency.

Rice (*Oryza sativa*) is a major crop and plant model, with significant progress of CRISPR/Cas-based studies. In a study conducted in 2013 by Shan et al., *Phytoene desaturase* gene (*OsPDS*) of rice protoplasts, targeted with CRISPR/Cas9, resulted in an efficient, targeted mutagenesis (15%) underlining the adaptability of CRISPR/Cas technique for the rice genome (Shan et al. 2013). Another study published in the same year presents a successful demonstration of type II CRISPR/Cas application in targeting the promoter region of the bacterial blight susceptibility genes (*OsSWEET14* and *OsSWEET11*) (Jiang et al. 2013). Since then there are various CRISPR/Cas-based studies conducted on the rice genome (reviewed in [Malzahn et al. 2017]).

11.8 Using Crispr to Modify Protein Families and Complex Genomes

While the identification of single mutants and their production is a methodology with a long tradition, one characteristic of plant genomes is the large amount of gene families, gene redundancies and polyploid genomes. Under these scenarios, the use of CRISPR/Cas has shown to be a major breakthrough as several genes can be engineered with a single construct, provided there is enough sequence similarity.

Abscisic acid (ABA) is an important regulator of environmental stress responses including but not limited to drought, salinity and heat stresses. ABA directly regulates the control of stomatal closure and organ growth. The pyrabactine resistance 1 (PIR1) and PYR-like (PIL) proteins form a family of fourteen members in the Arabidopsis genome acting as ABA receptors. In a seminal experiment using CRISPR/Cas (Zhao et al. 2018), a set of new mutations were stacked onto a previously sextuple mutant, thus obtaining a duodecuple mutant of PYR/PIL genes in Arabidopsis (Gonzalez-Guzman et al. 2012). This shows that gene families can be engineered to obtain

high-level mutant combinations that would require a very long time to be generated in form of single mutants, even in short-lived plants such as Arabidopsis.

Potato (*Solanum tuberosum*) is an important agricultural plant, ranking as third most important crop in the world with a long history of biotechnological approaches (Barrell et al. 2013). Potato is an essential crop due to its high amount of starch. Importantly, the commercial potato is highly heterozygous and autotetraploid. In a study, CRISPR/Cas system has been used to mediate interference on a gene encoding granule-bound starch synthase *(GBSS)* (Andersson et al. 2017). In another study, targeting of *ACETOLACTATE SYNTHASE1* (StALS1) resulted in the generation of multi-allelic mutagenesis (Butler et al. 2016).

Commercial wheat (*Triticum sp.*) comprises three genomes, but due to its extreme importance it has been targeted by several CRISPR/Cas-based studies, NHEJ-based mutation induced on *MLO* (mildew resistance locus) related to natural powdery mildew resistance (Shan et al. 2013; Wang et al. 2014). Another study shows the successful targeting of both Phytoene desaturase (PDS) which is key enzyme of carotenoid biosynthesis and inositol oxygenase (INOX) gene responsible from the oxidization of myo-inositol into glucuronic acid of *Triticussm aestivum.* The targeting resulted in the production of efficient incidences of indels (insertion & deletion), consequently suggesting the possibility of allohexaploid gene editing with CRISPR/Cas systems.

Camelina is an allohexaploid plant and an emerging crop for high-quality oil. The objective of gene modification studies in *Camelina* is to increase the oleic acid while decreasing the linoleic and linolenic acid content (Weeks 2017). A group of *Fatty Acid Desaturase 2 (FAD2)* genes play a role in both linoleic and linolenic acid biosynthesis. *FAD2* genes, located on three independent pairs of Camelina chromosomes, were targeted for mediated interference (Jiang et al. 2017). The analysis of T4 generation seeds shows an increase of 50% in the oleic acid levels while reducing the polyunsaturated fatty acid levels by 15%. Another study conducted by Morineau et al. (2017) created a large number of *FAD2* gene knockouts, obtaining successful knockout plants of all existing *FAD2* genes (reviewed in [Weeks 2017]).

Recently another approach was performed on the banana genome by Kaur et al. The banana gene phytoene desaturase *(RAS-PDS)*, involved in the pathway of carotenoid biosynthesis, has been targeted for interference mediation and the resulting 13 mutant lines have been examined for chlorophyll and carotenoid content (Kaur et al. 2018).

Oilseed rape (*Brassica sp.*) is a tetraploid plant which is important for its oil content. Two *ALCATRAZ* (*ALC*) homeologs of *Brassica napus* have been targeted with CRISPR/Cas9 to increase shatter resistance, an important issue causing up to 25% seed loss during preharvest (Braatz et al. 2017). It has been observed that the plants with disrupted *ALC* function are lacking the production of specialized silique tissues, leading to reduce disease resistance. The rapeseed plants with knocked out *ALC* function show a lower seed loss in the process of threshing. The targeted mutagenesis of *BnALC* homologs resulted in the mutation of four alleles in a single T1 plant using a single target sequence, indicating the possibility of simultaneous modification of different homoeologous gene copies in polyploid species.

11.9 Conclusions

Fruit and vegetable improvement, both concerning product quality and yield, is a major aspect of plant breeding. Advancements in the field of gene editing strategies allow scientists to rapidly obtain fast and efficient results compared to the traditional gene modification systems. The basic working principle and versatility enable CRISPR/Cas systems to be the most powerful gene modification tool since the beginning of plant breeding. As an example, the development of gRNA libraries increases the speed of the CRISPR/Cas application to discover certain functional genes or the regulatory elements. The development of online tools, providing pooled CRISPR libraries, speed up the process of advancements in this field. For instance, tomato CRISPR libraries have been generated based on the *Agrobacterium*-based T-DNA delivery technique for the generation of mutants for gene families (Jacobs et al. 2017). Consecutively, the web tools enabled scientists to target single genes on a certain plant genome while allowing the identification of the same gene on different species.

The identification of relationships among the traits of interest with selectable markers is crucial in order to improve the accessibility and rapidity of gene modification studies.

The importance of CRISPR/Cas system lies in the fact that it can be easily designed for different purposes such as visual identification of defined regions by the combination of CRISPR technique with fluorescent proteins as well as purification and isolation of proteins and nucleic acids associated with DNA or RNA (Tanenbaum et al. 2014; Fujita and Fujii 2013). Future studies should be carried out in order to enhance the efficiency of gene targeting applications in the field of plant breeding.

Acknowledgements This work was developed under project BFU-2017 88300-C2-1-R and FEDER.

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Chapter 12
Principles and Applications of RNA-Based Genome Editing for Crop Improvement

M. Nagaraj Kumar, V. V. Santosh Kumar, Archana Watts, and Viswanathan Chinnusamy

Abstract Ever increasing population demands highly productive agriculture and nutritive crops. Precise genome editing serves as a promising tool to meet out those demands by rapid crop improvement. CRISPR/Cas system and its latest versions are exhibiting its potentiality on targeted editing, single base substitution, multiplex editing of key genes for accelerating plant growth and development. In this chapter, we review the basic principles of CRISPR/Cas9 system, modifications of Cas proteins, delivery methods and applications. We also addressed the applications of this technique for elevating crop yield and increasing biotic and abiotic stress resistance. Additionally, we summarized the regulatory aspects of genome-edited crops in India as well as in other countries. Although CRISPR/Cas is successful, it has some technical limitations which are mentioned at the end of this chapter. Altogether, this chapter could provide an overall picture about CRISPR/Cas systems and their influence in plant science research.

Keywords CRISPR/Cas9 · Genome editing · Base editing · Multiplexing · Cas9 engineering · Cpf1 · Cas13 · gRNA-Cas9 delivery · GE crops regulation

12.1 Introduction

Crop cultivation had been originated through the domestication of plants of interesting traits such as desirable architecture, altered plant height, increased number of tillers and/or seeds, modified fruit size and shifted flowering pattern. These traits were altogether hand-picked by farmers and being grown with the aim of getting higher returns from their fields. On the other end, plant breeders introduced the genetic improvement through interspecific and intraspecific crossing of sexually propagated plants and also through spontaneous mutations for asexually propagated plants by

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G. Tang et al. (eds.), *RNA-Based Technologies for Functional Genomics in Plants*, Concepts and Strategies in Plant Sciences, https://doi.org/10.1007/978-3-030-64994-4_12

molecular breeding approaches (Sleper and Poehlman 2006). However, the mutations remained as the key to introduce genetic variability for aforementioned crop improvement processes. Random mutagenesis is the mother of all genome editing techniques as of till date but duration and efforts are the key limiting factors of developing mutated plants through natural mutagenesis. Generally, classical plant breeding strategies are time-consuming in terms of getting the best germplasm after crossing elite cultivars whereas genome editing snaps desired target as programmed and is efficient in terms of genome manipulation. So, the gear was shifted from molecular breeding to precision breeding in which targeted editing of genomes is the holy grail which allows rapid introduction of genetic diversity and accelerates variety development (Jansing et al. 2019b).

12.1.1 Genome Editing—As Molecular Scissors of Mutation

Genome editing is the targeted modification of gene(s) of interest through insertion, deletion, substitution in the genome to decipher gene functions. Site-specific double-strand breaks (DSBs) is the trigger for genome editing after which subsequent DNA repair is performed through error-prone non-homologous end joining (NHEJ) and error-free homology-directed repair pathways (HDR) (Zhang et al. 2017a). NHEJ is the most efficient and occurs in all stages of the cell cycle whereas HDR has low efficiency and occurs at S/G2 phases of the cell cycle (Mladenov and Iliakis 2011; Puchta 2005). HDR pathway is a highly precised technique for editing the target sites performed by utilizing a DNA template that is homologous to the upstream and downstream site of DSB (Jansing et al. 2019b). Unlike HDR, NHEJ causes rearrangement of chromosomes because of occurrence of DSB at multiple sites, followed by the fusion of inappropriate ends (Pacher et al. 2007). These mechanisms of DSB repair necessitate the way for targeted breaks at the specific sites for controlled manipulations of the genome. The advent of site-specific nucleases, as the programmable enzymes, solves the purpose of site-specific editing and the induction of DSBs (Jansing et al. 2019b).

12.1.2 Tools for Genome Editing

Genome editing approaches are quite relevant and having wide applications due to development and evolution of site-specific nucleases and precise editing of desirable targets. In general, the site-specific nucleases are composed of DNA binding domain which binds to the target and non-specific endonucleases which makes a snap at the target. These events initiate NHEJ or HDR pathway for DNA repair. NHEJ pathway for repair of DNA ends is non-specific due to the insertion or deletion of bases of different sizes whereas HDR remains specific because of the homology

template added as a donor DNA for the repair mechanism (Puchta 2005). Till date, the well-studied nucleases for genome editing are Zinc Finger Nucleases (ZFNs), Transcription activator-like effector nucleases (TALENs), Clustered regularly interspaced short palindromic repeat—Cas9 (CRISPR/Cas9) and the multiple variants of CRISPR associated protein 9 (Cas9).

12.1.2.1 ZFN (Zinc Finger Nucleases)

ZFNs are the first artificial nucleases that are used for genome editing. They consist of a specific domain for DNA binding called Zinc Finger motif and non-specific FokI nucleases attached to the C- terminal of zinc finger motif. Zinc fingers are the series of three to six repeats of 3 bp DNA sequence, together called zinc finger array. Two zinc finger arrays are designed individually that bind to forward and reverse strands of the DNA leaving the gap of 5–7 bp in the target sequence. The two different zinc finger array (5' to 3' and 3' to 5' terminals) are necessary for leading the dimerization of FokI which is necessary for its nuclease activity (Lloyd et al. 2005; Zhang et al. 2017a). Fok1 is the type IIS restriction enzyme from *Flavobacterium okeanokoites* (Kim et al. 1996). Despite being used in various crops such as corn, soybean and Arabidopsis, ZFNs have key limitations in multiplex editing, target selection and laborious cost of assembly (Zhang et al. 2017a).

12.1.2.2 TALENs (Transcription Activator-Like Effector Nucleases)

TALENs are the second generation of artificial nucleases comprising of specific target binding TALE effector domain (Transcription activator like) and non-specific FokI nuclease domain (Mahfouz et al. 2011). TALE domain consists of 33–35 tandem repeats of amino acids which are conserved except two residues, called as RVD (Repeat Variable Di-residues) (Bogdanove et al. 2010). Like ZFNs, TALENs also contain FokI nuclease attached to the two monomers of TALE domains. The spacer length between TALEN monomer in the target is 15–20 bp that is higher than that of ZFN. The ease of making the assembly lets TALEN as a better tool for genome editing than ZFN. Similar to ZFN, TALEN also has a few limitations in terms of abundant targets, multiplexing and cost of assembly (Zhang et al. 2017a). Despite TALEN having better efficiency in comparison to ZFN, the number of reports of genome editing are very few for both classes of artificial nucleases. This could be due to a few reasons *viz*., higher number of tandem repeats for binding with the target, experimental conditions and the choice of targets (Jansing et al. 2019b). The most common key limiting factor for both ZFN and TALEN is the stringent requirement of dimeric guide sequences of protein for a single target. This technique hampers the aim of doing multiplexing in which DSB occurs at multiple sites of the target (Kannan et al. 2018).

12.1.2.3 CRISPR/Cas System (Clustered Regularly Interspaced Short Palindromic Repeats)

CRISPR/Cas9 is the most flamboyant of the third generation of sequence-specific nucleases and is being extensively studied for enabling DSBs at desired site of target in the genome. CRISPR was first discovered in *Escherichia coli* as the DNA fragment with short repeats inter-twined with spacers derived from invading bacteriophages. Initial evidence of involvement of CRISPR in adaptive immunity was found out through the addition or deletion of these spacers in the bacterial system (Barrangou et al. 2007). Two or three nucleotides among the spacers acquired through phage invasion in bacteria served as a protospacer motif (PAM), crucial for the recognition of targets by the nucleases. Cas9 is the associated nuclease with CRISPR, located proximal to CRISPR locus (Ishino et al. 1987; Jansen et al. 2002). This system consists of a single effector Cas endonuclease and chimeric guide RNAs which together form ribonucleoprotein complex (RNP) to justify its role. Chimeric RNA acts as a single guide RNA (sgRNA) and consists of CRISPR RNA (crRNA), composed of target-dependent nucleotides, and trans-activating crRNA (tracrRNA), which interacts with both crRNA and Cas9 nuclease (Jinek et al. 2012). Cas nuclease contains HNH and RuvC-like domains. HNH binds to the complementary strand of guide RNA whereas RuvC-like domain binds to the non-complementary strand of the target. After binding, Cas9 endonuclease produces double-strand break (DSB) by blunt end cutting at upstream of PAM (Makarova et al. 2015; Jinek et al. 2012). CRISPR system is relatively easier and faster to design, as only sgRNA sequence has to be designed for pairing with the target gene and there is no requirement of any protein modifications like ZFNs and TALENs. Hence, these features basically make CRISPR/Cas system user-friendly technology in comparison to ZFNs and TALENs (Jinek et al. 2012; Bao et al. 2019).

After an initial report of CRISPR/Cas in plant system in 2013, CRISPR/Cas system has burgeoning fame and overwhelming output because of its applications in all areas of plant biology. Recent paper from Science reported that the number of publications and patents related to CRISPR are increasing steadily. Of which, the USA and China are the leading giants in the current scenario of blooming CRISPR applications in the field of mammalian and plant sciences. As of 2018, USA ranks top in terms of publishing papers (898) followed by China with a marginal difference (824). Japan, UK, Germany and Canada followed the consecutive places of publishing their CRISPR research (Cohen 2019). For more information on the chronological developments of CRISPR from its first report to the recent application, please refer to the review article by Razaaq et al. (2019). The detailed workflow of CRISPR is furnished in Fig. 12.1. The current chapter will focus on the multi-faceted features of CRISPR technique and its development for tremendous success in the field of plant biology.

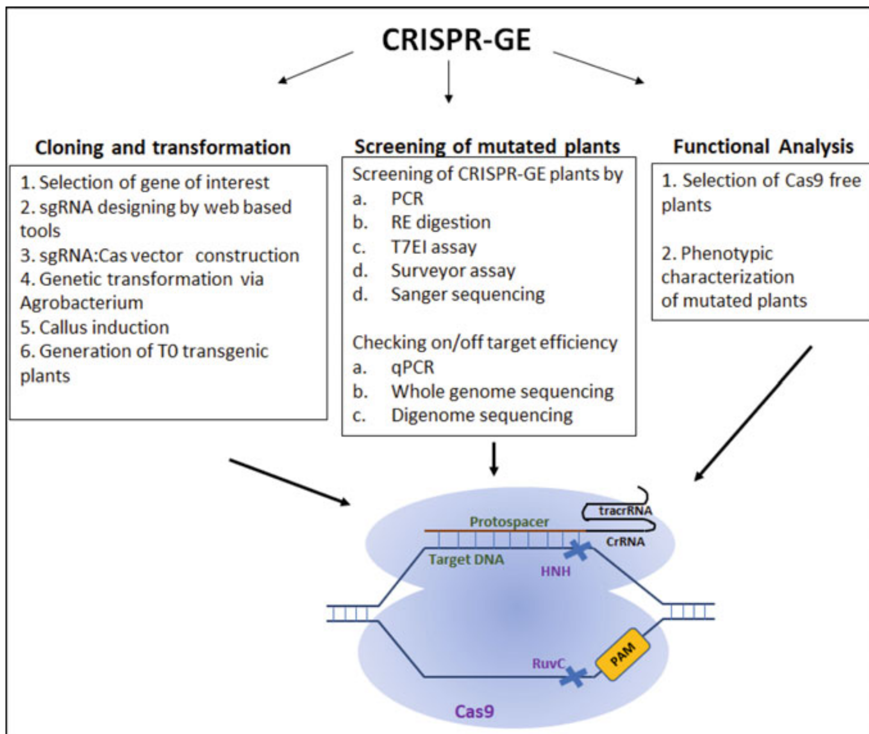

Fig. 12.1 Summary of workflow for generating CRISPR-GE (genome editing) crops. Three major steps are described here primarily involved in GE of crops. First, the workflow starts with construction of gRNA:Cas9 and transferring gRNA:Cas9 into plants. Second, confirmation of edited plants through various methods. Third, phenotypic analysis of gene edited plants

12.2 CRISPR/Cas System—A Wide Horizon of Genome Editing

12.2.1 CRISPR/Cas9

CRISPR is an adaptive defence system in prokaryotes to combat against foreign pathogens. CRISPR system has classified into six major types, *viz*., type I to type VI. Each system has its own signature single Cas protein (in case of type II, V, VI) and multiple Cas proteins (in case of type I, III, IV) (Shmakov et al. 2017; Koonin et al. 2017). Of which, type II system encompasses the Cas9 protein from *Streptococcus pyrogenes* and SpCas9 is one of the mostly used nucleases for genome editing in plants (Makarova et al. 2015). As mentioned in the previous section (see also in 1.2.3), the CRISPR system is engineered based on type II Cas9 with tailored sgRNA comprised of fused crRNA and tracrRNA. SpCas9 is programmed to make DSBs at three bases upstream of PAM sequence of NGG in which N could be any one of the

four nucleotides preceded by N20 nucleotides of the gRNA sequence. Recently, it was found that SpCas9 engineered version, SpCas9-NGv1 could target NG PAMs in rice and Arabidopsis (Endo et al. 2019). The PAM sequence of Cas9 from *S. thermophiles* is 5'-NGGNG or 5'-NNAGAA whereas Cas9 from *Neisseria meningitidis* recognize the PAM of 5'-NNNNGATT (Garneau et al. 2010; Zhang et al. 2013; Gasiunas et al. 2012). Both sgRNA and Cas9 cassettes are introduced into plants by Agrobacterium-mediated transformation. Several features need to be considered for the successful genome editing of which the choice of promoters for gRNA and Cas9 is essential. Generally, gRNA is expressed by either U3 or U6 promoter-driven by RNA polymerase III whereas Cas9 is expressed by either ubiquitin or 35S promoter-driven by RNA polymerase II. The U6 and U3 promotors have definite transcription start nucleotides like G and A, respectively. So, the consensus guide sequences are G(N19)-PAM and A(N19)-PAM for U6 and U3 promoters, respectively (Nekrasov et al. 2013; Feng et al. 2013; Mao et al. 2013; Xie and Yang 2013; Miao et al. 2013; Jiang et al. 2013). Also, Cas9 with nuclear localization signal ensures the likely integration of the construct with the plant genome (Belhaj et al. 2013). Besides, the secondary structure of both gRNA and the target, the codon usage of Cas9 in plants, GC content of both gRNA and the target altogether influence the targeting efficiency of CRISPR/Cas9 in plants (Ma et al. 2015b).

During the initial stages, CRISPR applications had resulted in low efficiency in editing, therefore much improvements were continuously made later to improve its functionality through the selection of proper vector as well as the target, efficient construction of gRNA-Cas9 cassette, and improvement of delivery methods to plants. gRNA selection is one of the key steps for CRISPR-mediated genome editing, for which approximately 22 softwares have been developed within six years from 2013–2019 (Razzaq et al. 2019). Recent report (Gerashchenkov et al. 2020) has indicated the existence of 100 programmes to design gRNAs for CRISPR/Cas systems. Most of them are free to access and can also predict off-targets and secondary structures. In plants, for constructing efficient gRNA cassette, overlapping PCR or adapter ligation method has been used. For designing Cas9 cassette, plant-based codon should be used to improve the editing process in plants (Xie and Yang 2013; Fauser et al. 2014). Improved expression vectors have been developed by using single polymerase II and dual polymerase II driven gRNA: Cas9 cassettes. In the case of single pol II, both the guide RNA and Cas9 were expressed by a single promoter in the vector whereas, in dual pol II, different promoters drive their expression (Lowder et al. 2015). Owing to ease of developing CRISPR cassette, it has widespread applications in most of grain crops like rice, wheat, maize, sorghum, barley and fruits like tomato, sweet orange, apple and also in other crops like cotton, lettuce, soybean, citrus, lotus, petunia including mushroom and Arabidopsis. For all the references of above crops, please go through the review published in critical reviews in biotechnology (Bao et al. 2019).

12.2.2 CRISPR/Cas12a (Cpf1)

This is the next generation of Cas9 advancement into Cas12a, otherwise called Cpf1, the name was derived from *Prevotella* and *Francisella* bacteria. It is a monomeric protein belongs to the type V category of CRISPR system. It recognizes T rich PAM sequences like 5’ TTTN 3’ or 5’ TTN 3’ located at the 5’ end of the target and makes a staggered cut with overhanging five nucleotides at 5’ end of the PAM. This sticky cutting results in the loss of 6–13 bp, causing a larger deletion than Cas9 (Tang et al. 2017). In this system, crRNA (42 nucleotides) guides Cpf1 and cleaves the target without the need of tracrRNA as in sgRNA-Cas9 (Zetsche et al. 2015). Besides, Cpf1 has dual functions as a nuclease and as an RNAase where it cleaves at the target site and processes the pre-crRNA to mature crRNA, respectively (Dong et al. 2016; Fonfara et al. 2016). This dual role of Cpf1 has nodded off the usage of separate promoters for each gRNA while multiplexing. Another salient feature of Cpf1 is mainly its versatility by which it can be deployed in multiplexing, base editing and epigenetic modifications. The notable advantage is that Cpf1 generates low off-targets compared to Cas9 (Bayat et al. 2018; Zaidi et al. 2017). In plants, heritable mutations generated by Cas12a was first reported in rice and tobacco. Increased efficiencies of both FbCpf1 and LbCpf1 were observed in these studies (Endo et al. 2016; Xu et al. 2017). This could be mainly because of the reasons such as snapping by Cas12a leads to editing with HR because of the generation of overhangs away from PAM that promotes the repair preferably through HR than NHEJ (Begemann et al. 2017). The features of gRNA such as GC content, melting temperature, free energy and the attributes of the target significantly determine the efficiency of mutations generated by Cas12a (Safari et al. 2019). CRISPR-DT was the first web-based tool that helped to generate gRNA sequences for using Cas12a. CRISPR Inc is another web tool, works simple and rapid for gRNA designing, based on recent annotations and covers the pre-searched targets of Cpf1 in the complete genome of twelve organisms (Zhu and Liang 2019; Park and Bae 2017). As of now, there are three Cpf1 systems available for genome editing in plants *viz.*, FnCpf1, LbCpf1 and AsCpf1 (Tang et al. 2017; Xu et al. 2017; Wang et al. 2017b). A comparative diagram of Cpf1 and Cas9 functioning are furnished in Fig. 12.2.

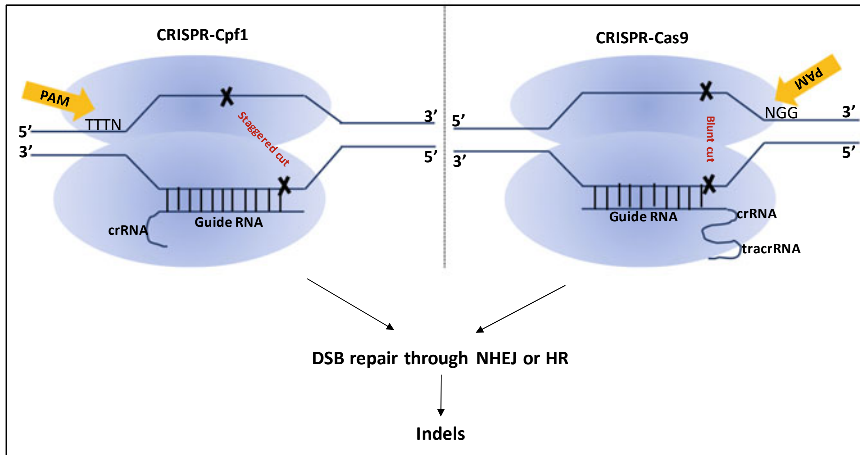

Fig. 12.2 Schematic diagram of two major CRISPR/Cas systems in plants. This figure depicts the key differences between Cas9 and Cpf1 system of gene editing. Cas9 makes blunt cut close to its PAM whereas Cpf1 makes staggered cut away from its PAM motif. It is also important to note the CRISPR RNA length is minimal in case of CRISPR/Cpf1 system

12.2.3 CRISPR/Cas13

Cas13 is the newest entry to CRISPR systems and it specifically targets cytoplasmic RNAs. This nuclease acts particularly on RNA through its catalytic activity of HEPN domain. Similar to Cas12, Cas13 also processes pre-crRNA, independent of tracrRNA, through its catalytic activity of Helical1 domain (Shmakov et al. 2015; Abudayyeh et al. 2016, 2017). In addition to mRNA, Cas13 also targets non-coding RNA which is very promising because of the key role of non-coding RNAs in gene regulation, protein translocation and splicing (Abudayyeh et al. 2017). These RNAs are the key targets of Cas13 *in vivo*, it provides ample chance of editing with inducible or tissue-specific promoters to avoid lethality due to the complete gene knock out (Schindele et al. 2018). Also, RNA virus infection and suppression were mitigated by exploiting the RNA targeting ability of Cas13 given the fact that RNA is the core of the majority of infective particles of the plant viruses. Since Cas13 targets mRNA, its likely applications in the field of post-transcriptional repression, mRNA transport, RNA binding proteins among others were evident (Abudayyeh et al. 2017).

12.2.4 Base Editing

The change in the single nucleotide base from the specific site of the genome without disruption of a gene, that leads to a notable phenotypic output is called base editing. This could be performed precisely through CRISPR/Cas system confining it to the

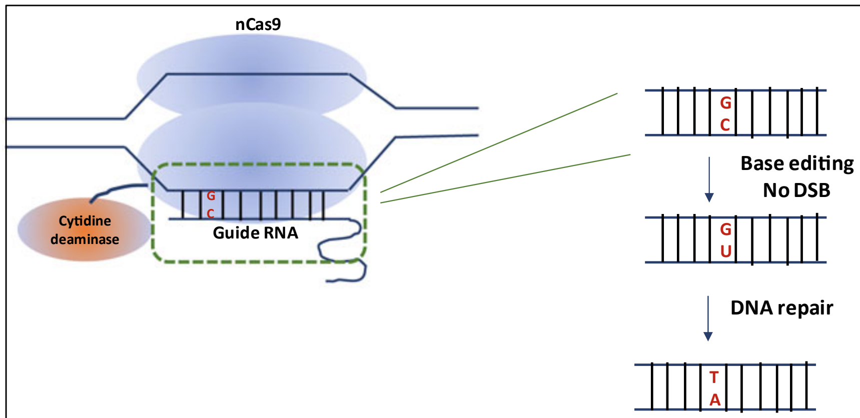

Fig. 12.3 Schematic diagram depicting the principle of base editing. An example of base editing in this figure shows that how the concept of single nucleotide change occurs through cytidine deaminase-fused nCas9 system

target in which the single base has to be edited. Base editing facilitates the direct change of nucleotides that are irreversible and causes promising genetic variants for crop improvement (Mishra et al. 2020). The limitations of double-strand break repair through NHEJ and low efficiency of HR in CRISPR raised the need of base editors to create point mutation at the desired target without double-strand break (Komor et al. 2016). Cytidine deaminase and nickase are the key components of the base editors in which the former changes the cytidine base to uridine and the latter changes Cas9 into nCas9 which is semi-active nuclease due to the mutation of D10A in one of its nuclease domains. Like CBE, ABE is designed to base edit the desired targets in plants by fusing adenosine deaminase with nCas9. The combination of these enzymes creates mismatch repair which resulted C: G altered to T: A base pair (Lu and Zhu 2017) (Fig. 12.3). Base editing has been standardized and employed in crops such as rice, wheat and maize (Zong et al. 2017). Interestingly, tRNA based adenosine deaminase (ABE) has been recently reported to change T: A to C: G which was difficult to alter previously. Through this adenosine deaminase application, 7.5% base editing in protoplasts and 59.1% base editing in stable transgenic rice plants were reported (Gaudelli et al. 2017; Li et al. 2018b). The readers are suggested to go through the information for the full list of base editors employed for crop improvement that has been updated in the review of Mishra et al. (2020). Recently, advanced base editor 3 was developed which has improved version of cytidine deaminase derived from rat (APOBEC1) and modified PAM sites to increase base editing efficiency (Hess et al. 2017). Similarly, APOBEC3A and AID from human and PmCDA1 from Lamprey were also fused with nCas9 and has been used in plants as base editors (Razzaq et al. 2019).

12.2.5 Multiplexing

Cellular development and growth in plants are usually governed by multiple genes. Also, few members of the gene families contribute to the important traits of interest in plants. Because of the natural phenomenon of association of several genes for expression of a single trait, more than one or many genes have to be edited or knocked out at multiple sites to study their association with the trait of interest. Multiplexing mediated through CRISPR/Cas9 involves the designing of multiple sgRNAs with single or multiple promoters expressed in a single vector system (Liu et al.2017a; Xing et al. 2014). Multiplexing technically can be categorized into different types based on the number of gRNA, promoters and the linkers attached in the cassette. Multiple gRNAs can be delivered as individual cassettes or as polycistronic cassettes by Cas9 nucleases or by its variants. However, multiple gRNAs with separate promoters is the typical multiplex which has been used so far. Some studies reported that multiple gRNA can be expressed as a single transcriptional unit in which functional gRNAs were individually generated by supplied ribozyme or by their own tRNA transfer system (Gao and Zhao 2014; Xie et al. 2015). Several approaches are used for multiplexing gRNAs by following one of the three methods *viz*., golden gate assembly, polycistronic tRNA-gRNA system, ribozyme cleaving system and target-adaptor ligation (Lowder et al. 2015; Ma et al. 2015b). Instead of designing multiple cassettes of gRNAs for multiple editing, CRISPR-Cpf1 provides an easy platform for multiplexing. Cpf1 only needs single, direct short repeat spacer sequence (DR) which is processed by Cpf1 itself into functional crRNA units (Zhang et al. 2017a). Based on this strategy, Wang and the team have reported the efficacy of LbCpf1 and FnCpf1 in the editing of six different sites of three genes in rice such as Os*PDS*, Os*BEL* and Os*EPSPS.* Both variants of Cpf1 caused multiplex editing with their mature DR sequences and among them, LbCpf1 exhibited better editing than FnCpf1 (Wang et al. 2017b). Recently, SSTU (Simplified Single Transcriptional Unit) system was developed for multiplexing in rice to express FnCpf1 or LbCpf1 or Cas9 in which both the nuclease and crRNA are expressed under single Pol II promoter without any additional modules in the multiplex cassette (Wang et al. 2018a). Multiplexing was initially focussed for traits like yield increase and herbicide resistance but to date its application has been expanded from hormone perception to molecular farming (Najera et al. 2019).

12.2.6 CRISPR—Off-Targets

The specificity of CRISPR completely relies on 20 bp gRNA sequences complementary to the target of interest. Given the facts that the entire genome of the target is larger and Cas9 cleaves the target-like sequences instead of the right targets, leading to off-targeting of the CRISPR system, reported in several studies so far (Fu et al. 2013; Hsu et al. 2013; Pattanayak et al. 2013). Non-specificity arises mostly because

of the mismatches of 8–10 bp near to 3' end of gRNA (seed region) and mostly mismatches close to the 5' end are least bothered. Also, noted that PAM should follow immediately next to 20 bp of gRNA without any additional sequences (Hsu et al. 2013). This non-specific binding can mainly be avoided by designing the utmost specific gRNA sequence with the probability of fewer off-targets; identified by using web tools such as Cas-OFFinder, CRISPR-P, CRISPR-GE, sgRNAcas9, CRISPR design, E-CRISPR and CHOPCHOP. Additional strategies to avoid off-targeting are through using- truncated gRNAs with less than 20 bp, extra nucleotides like GG added to 5' end of gRNA sequence (Fu et al. 2014; Cho et al. 2014), and through using paired Cas9 nucleases, dCas9-*Fok1*, split Cas9, high fidelity Cas9 variants such as eSpCas9 1.0, eSpCas9 1.1 or using an engineered Cas9, SpCas9-HF1 (Komor et al. 2017; Kleinstiver et al. 2016a; Slaymaker et al. 2016). Despite, much attention has been given to minimize off-targeting in human genome editing for therapeutic applications, this is not worrisome in plants because the off-targets could be easily managed by segregation of allele through generations and getting rid of through the back crossing that will efficiently remove secondary mutations due to off-targeting (Schulman et al. 2020). It was also interesting to report that CRISPR/Cpf1 produces less off-targets compared to CRISPR/Cas9, which was consistent with the reports from animal studies (Kleinstiver et al. 2016b).

12.2.7 Delivery Methods of CRISPR Cassette

The delivering of CRISPR constructs into plant cells is as important as designing the construct for generating the edited plants, because the delivery method is one of the crucial deciding factors of CRISPR efficiency. CRISPR/Cas components are being transferred into plant cells mainly by *Agrobacterium*-mediated T-DNA transformation, particle bombardment and protoplast transfection methods. The first two methods are used for generating edited plants and the last one used for transient expression. *Agrobacterium* is the most commonly used tool for the transformation in general and also for editing constructs because of its high degree of T-DNA integration with the host genome. *Agrobacterium* is also used for the transient transformation of CRISPR constructs which resulted in low efficiency of both on and off-target mutations whereas stable transformations provided good expression of genome editing components and yielded a high frequency of on-target mutations (Jansing et al. 2019a; Chaparro-Garcia et al. 2015). The main lacuna of *Agrobacterium* transformation is the host specificity since some plant species, especially monocots that are recalcitrant to *Agrobacterium*-mediated transformation. Therefore, the second preferred method for transformation is the particle bombardment in which gold/tungsten particles are coated with DNA, RNA, protein, RNP and accelerated by gas pressure systems into plant cells (Sanford 1990). The quality and quantity of integration are much lower in this method compared to *Agrobacterium*-mediated transformation. However, it is advantageous that the broad host range of species are covered by particle bombardment (Verma et al. 2014).

Compared to plant systems, animal studies are using different methods for the delivery of CRISPR constructs because of the lack of cell wall in animal cells. If plant cell wall is removed, then multiple other methods for the delivery of CRISPR constructs are quite feasible in the plant system as well. Protoplast transformation is one of its kind to transform plants for genome editing with the help of PEG (polyethylene glycol) that helps the construct to permeabilize through plasma membrane (Darbani et al. 2008; Potrykus et al. 1998). Since protoplast transformation is a physical method, no specialized vectors are needed to transform the organism and also, multiple plasmids could be transformed at the same time resulting in transient or stable transformation with high frequencies (Baltes et al. 2017). Recently, by using magnetic field exposure, the construct coated with magnetic particles are directed to pollen grains for transformation. This could be applied for CRISPR constructs to increase their broad host range for transformation (Zhao et al. 2017). Recently, many variations in *in vitro* and *in planta* transformation of both crop plants and Arabidopsis with CRISPR-Cas constructs have been addressed in detail (Zlobin et al. 2020).

12.2.8 Engineered Cas9 Modifications

The generation of knock out mutants by CRISPR created a huge wave in the field of functional genomics (Decaestecker et al. 2019). However, this method has its limitations because of pleiotropic and lethal effects caused by loss-of-function of single gene. Although different plant species encode large number of genes, only small percentage of genes are important for the plant functions. For example, only 10% of ~25,000 genes in Arabidopsis are indispensable (Lloyd et al. 2015). Therefore, knock out of genes through CRISPR/Cas system should be customized based on the cell type, tissue type and organ type which is essential for reaping the complete benefits of this technology. Other than plant science, researchers working on mammalian systems have already demonstrated that modifications and/or fusion protein attachments of Cas9 have resulted in the tissue-specific knock out of gene of interest. One such example is that targeted knock out of *wingless* and *wntless* genes in Drosophila germ cells led to the generation of adult files whereas its non-specific overall knock out caused lethality (Port et al. 2014). Likewise, in plants, xylem specific promoter *NST3/SND1* was used to drive Cas9 expression in xylem cells to target *HCL* which resulted in decreased lignin content only in the specific cells (Liang et al. 2019).

2.8.1 CRISPR-TSKO (Tissue-Specific Knock out) is a new toolset that arrests gene activity in the tissues of interest leading to subset level genome editing (Ali et al. 2020). This technique is based on Golden gate and modified Green Gate vector technologies and designed for different cells, tissues and organ types for which Cas9 is driven by the respective tissue-specific promoter and attached with a fluorescent protein. By using this approach in Arabidopsis, nine different genes were targeted with four different tissue-specific promoters driving Cas9. Among the genes, *PDS3, YDA, CDKA1* are essential for plant growth and reproduction and whose ubiquitous knock out caused lethality whereas the TSKO approach yielded

viable plants from those mutants. This system allowed to study gene function in a spatial-temporal manner which was unlikely earlier due to the pleotropic effects of the loss of gene function (Decaestecker et al. 2019). Despite being useful for tissue-specific knock down of pleotropic genes, CRISPR-TSKO technique invariably depends on the promoters which may be leaky in neighbouring cells other than the targeted ones (Ali et al. 2020).

2.8.2 Cell type specific promoter—Genome editing in plants should be heritable in terms of the targeted mutation by CRISPR/Cas system. Most of the ubiquitous promoters such as 35S has been used to drive Cas9 expression, not providing good expression in meristematic and reproductive tissues (Ge et al. 2008). So it is necessary to use germ line-specific promoters such as egg cell-specific promoter EC1 and meristem-specific promoters such as CDC4 and, CLAVATA3 for heritable mutagenesis (Mao et al. 2016; Miki et al. 2018). Moreover, egg cell-specific promoters are preferred for DSB repair through HR due to the availability of donor template at higher concentration.

2.8.3 Cas9-PF—Generally, the stable integration of Cas9 and sgRNA have led to high-efficiency editing in plants. At the same time, retrieving edited plants free of Cas9 and gRNA is also important for CRISPR crops particularly for the concerns related to GMO regulations. The traditional methods of screening for Cas9-free plants require creation of T1 or T2 generation, and thereby is a time-consuming process. For generating edited plants without any background, Cas9-PF was developed in tobacco by Liu and team (Liu et al. 2019). In their work, they co-expressed *PAP1* (production of anthocyanin pigment 1) and *FT* (flowering locus T) in Cas9 cassette. *PAP1* served as a phenotypic marker to ensure the presence of CRISPR/Cas9 in T0 or T1 generation for selection. *FT* accelerated the breeding cycle for faster advancement into next generations. This PF cassette with Cas9 was used to target *E1F4E*, a recessive resistance gene to Potato virus Y in tobacco. Cas9-PF accelerated the process to get transgene free edited plants in a short time with increased efficiency.

2.8.4 Cas9-versions—Till now, many Cas9 variants and Cas9 from different bacterial sources are available for increasing the specificity as well as to enhance the editing efficiency. The best Cas9 for targeted knock out was studied by comparing the efficiency of different Cas9 on the same target. Cas9 from *Streptococcus pyogenes* (SpCas9), *Staphylococcus aureus* (SaCas9), *Francisella novicida* (FnCas12a), *Lachnospiraceae bacterium* (LbCas12a) and Cas9 engineered versions like eCas9 1.0, eCas9 1.1, eSaCas9 and xCas9 3.7 (Raitskin et al. 2019) in tobacco and Arabidopsis. The result indicated that SaCas9 has the highest editing efficiency. However, it may not be consistent because Cas9 activity depends on experimental conditions and its expression relies on promoters. Interestingly, temperature has been found to play a role in enhancing Cas12a activity (Moreno-Mateos et al. 2017; Schindele and Puchta 2020).

Besides its nuclease activity, Cas9 could be harnessed for different applications other than editing. The nuclease activity could be inactivated by introducing alanine substitutions in its catalytic sites at HNH and RuvC domains (Sapranauskas et al.

2011). Then the inactivated Cas9 variants (dCas9) have been used as a binding scaffold for attaching effector proteins for different purposes such as transcriptional activation, repression, histone methylation, and demethylation and mRNA transport and localization studies as well. For instance, in tobacco, *PDS* gene was transcriptionally tuned by fusing C terminus of dCas9 with the TAL domain or SRDX domain to activate or repress *PDS* expression, respectively (Piatek et al. 2015). Similarly, dCas9 is attached with fluorescent proteins to visualize the trajectory of target proteins and with demethylase to generate epigenomic modifications (Anton et al. 2014; Maeder et al. 2013). dCas12 and dCas13 were also used for multiple purposes, as mentioned above for inactivating RuvC domain of Cas12 and HEPN domain of Cas13, respectively (Platt et al. 2014; Liao et al. 2017). Hence, for successful use of CRISPR-based genome editing, a number of parameters need to be standardized. In Fig. 12.4, all essential factors for successful CRISPR are depicted.

2.8.5 Prime editing—This technique has recently taken genome editing to another level of success by introducing insertion, deletion and base to base conversion without the requirement of DSB and donor DNA template (Anzalone et al. 2019). In this novel approach, gRNA is replaced by pegRNA (prime editing gRNA) which drives

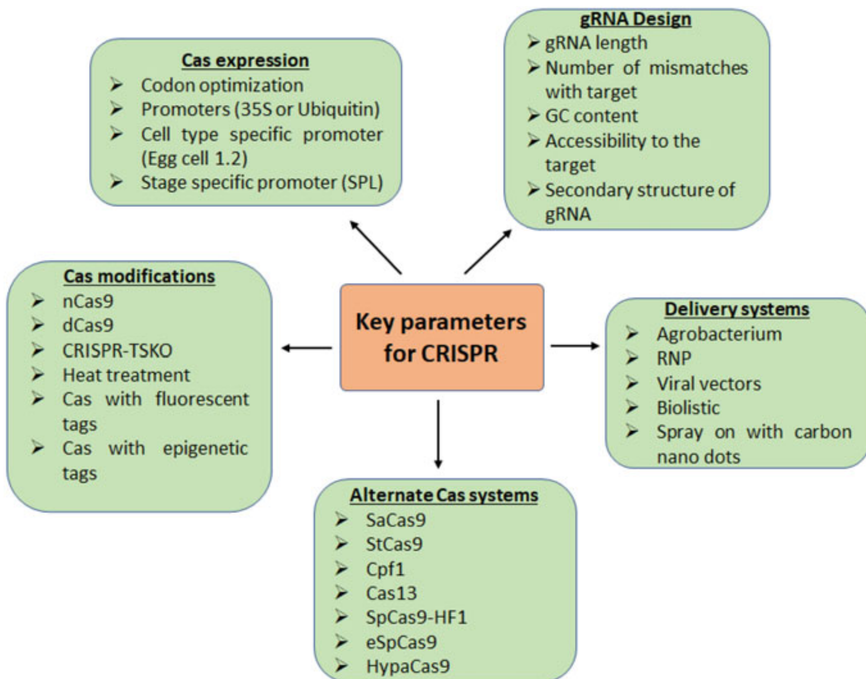

Fig. 12.4 Essential parameters for a successful CRISPR-GE experiment. This schematic diagram illustrates most of the key points for generating gene-edited plants through CRISPR system. It throws multitude of options from gRNA designing to delivery systems according to one's research directions

nCas9 fused with RT (reverse transcriptase). pegRNA contains PBS (primer binding region) which serves as template for RT to introduce mutations in the target. Lin et al. (2020) have firstly reported the prime editing application in plants by finding out the possibility of generating all types of base substitutions, insertions up to 15nt, deletions up to 40nt in the target genes of rice. Prime editing has been remained as more efficient and target-specific method than other editing technologies due to likely hybridization between the target and pegRNA which is probably higher at many places (Marzec and Henzel 2020).

12.3 CRISPR—For Revamping Plant Growth and Development

The advent and then development of CRISPR technologies has stirred basic plant biology research through its application towards crop improvement or new variety release. The molecular mechanisms CRISPR module have already been discussed in previous sections of this chapter, next let us see the variations of CRISPR modules and its applications in the following sections. Since its discovery in 2012, CRISPR application has been expanded to almost all of the major crops. Even, recalcitrant crop species which were difficult to transform, become amenable to gene editing because of the advancements in modulating Cas9 nuclease activity, its specificity and variants, base editing, and most importantly through novel delivery methods. In this section, we have attempted to uncover significant examples of applications of CRISPR in crop yield and resistance to biotic and abiotic stresses.

12.3.1 Yield and Grain Quality Enhancement

Yield is one of the key foci of plant science research as it is the ultimate contributor to sustained food security. Yield is a multi-genic quantitative trait, so knocking out single gene will not fetch desirable results. This creates the necessity to opt for multiple gene knockouts by CRISPR-based technologies. In other way around, knocking out negative regulators of yield through CRISPR, substantially contributed to yield advancement. Genes regulating tiller numbers, panicle size, grain size, grain weight in rice and wheat were targeted to increase the yield. Also, multiplex editing of three genes associated with grain size and weight in rice such as *GS3, GW2* and *GW5* has been achieved. Similarly, early heading genes, *Hd2, Hd4* and *Hd5* were targeted in rice since heading date is an important trait contributing to rice yield (Liu et al. 2016c, 2017b, c; Xu et al. 2016). CRISPR was also deployed in hybrid rice breeding as hybrid rice yield is 10–20% higher than inbred lines. Thermosensitive gene, *TMS5*, edited through CRISPR/Cas9 to develop 11 new lines of TGMS (thermosensitive genetic male sterile) *indica* rice. Likewise, PGMS lines are mutated by

targeting *CSA* gene in *japonica* rice (Zhou et al. 2016; Li et al. 2016b). Flowering is also a key trait directly associated with seed set and thereby yield. But this trait is mostly influenced by day length which is determined by its geographical distribution. In soybean and tomato, *FLOWERING LOCUS T* (*FT*) and *SELF PRUNING 5G* (*SP5G*) are mutated by CRISPR to alter day length-dependence and increase crop distribution in different areas (Soyk et al. 2017; Cai et al. 2018). In maize, narrowing the leaf angle affects the light-harvesting nature of maize leaves. *ZmLG1* gene was targeted by CRISPR/Cas9 and DTM line T0 (Desired Target mutator) and transferred to six different maize lines through hybridization to get LG1 edited maize plant. This study showed the application of genome editing in important crops like maize which needs a laborious method of tissue culture and plant transformation (Li et al. 2017a). Pedigree analysis and whole-genome sequencing enabled identification of around 57 yield-associated genes in IR8 rice variety and further, through Cas9 and dCas9 editing approaches, phenotypes of the most of the genes were found associated with the yield (Huang et al. 2018). These studies provided strong evidence of application of CRISPR in improving major yield traits of cultivated crops.

Quality, remains as an another economic aspect of crop plants and a key trait of focus in enhancing food production. As of now, several studies using CRISPR has shown improvement in terms of palatability, fragrance, storage and nutrition. For instance, in rice, eating and cooking quality increased by targeting *Waxy* gene. *SBEIIb* was mutated through CRISPR/Cas9 to reduce starch by enhancing amylose/amylopectin ratio. Likewise, starch in potato was reduced by targeted editing of *GBSS* (Zhang et al. 2018a; Andersson et al. 2017). Given that high concentration of PUFA (polyunsaturated fatty acid) decreases the oil quality and causes health concerns, *FAD2-1A* and *1B* were edited through CRISPR/Cpf1 to increase both yield and oil content in soybean (Kim et al. 2017). Gluten allergy is a serious concern causing celiac disease to the people who are dependent on wheat as their staple food. CRISPR/Cas9 alleviated the effect of gluten that is encoded by the α-gliadin gene family and produced low gluten wheat (Sánchez-León et al. 2018). So, the quality improvements in crops by CRISPR techniques keep continuing with the recent additions of high oleic acid content in *Brassica napus*, longer shelf life of tomato and higher level of lycopene in tomato (Okuzaki et al. 2018; Li et al. 2018c, e).

Fragrance in crops is one of the important qualities that has huge market demands. Fragrant rice was generated by targeting *OsBADH2* which produced 2AP (2-acetyl 1-pyrolline) compound, responsible for the fragrance. Both TALEN and CRISPR/Cas9 methods have been deployed to generate fragrance in Zhongua 11, from China (Shao et al. 2017). Seed longevity is an essential trait affected due to poor storage qualities in rice grains. *LOX* (lipoxygenase) genes are responsible for grain deterioration and act as negative regulators of seed longevity. TALEN approach mutated *LOX3* and caused an increase in the storage life of rice grains (Ma et al. 2015a).

Malnutrition is a seriously emerging problem arising due to the consumption of food deficient in essential proteins, energy, vitamins and minerals. It has been reported that 24,000 people died per day due to malnutrition (Potrykus 2008). So, it is the need of the hour to alleviate this problem through CRISPR. Predominantly, for more

than half of the world population, rice supplies most of the nutrients and calories. Despite being a rich source of carbohydrates, it lacks key essential amino acids such as lysine and tryptophan in its grains. This necessitates the need to improve the nutritional qualities of major crop plants. Several studies have reported the transfer of the seed storage and ferritin genes into rice by transgenic approaches but still this area is incomplete and needs lot of investigation. Interestingly, cadmium tolerant rice variety was generated through knocking out *OsNramp5*, a metal transporter, that controls the accumulation of cadmium in rice grains (Tang et al. 2017). Poor digestibility in sorghum is caused by the compound, Kafirin, which is encoded by *k1C* genes. This compound causes protein body accumulation and the grains become devoid of essential amino acid, lysine. *k1C* was targeted by CRISPR/Cas9 which increased the consumption and nutritive value of the crop. Similarly, polyphenol oxidase (*PPO*) gene in button mushroom (*Agaricus bisporus*) was edited to avoid browning, thereby increasing its market value (Li et al. 2018a; Waltz 2016).

12.3.2 Tackling Abiotic Stresses

Abiotic stress is one of the major limiting factors affecting plant growth and yield. Even though burgeoning publications have been out in the field of stress biology, the effective solution to the problems of drought, salt and cold stresses remains fragmentary. This could be mainly because stress tolerance is a complex trait regulated by a multitude of signaling pathways, regulated by several regulators. After the advent of CRISPR/Cas system, the development of efficient crops to withstand against adverse climatic conditions, is getting close to reality. For instance, ABA is a well-known stress hormone that controls several stress signaling pathways. It is perceived by PYL receptors, which is a 13 membered gene family in rice. Knocking out those 13 genes in rice by CRISPR/Cas9 resulted in increased rice productivity as well as plant growth. Among the mutated lines, the group I (edited *PYL1* to *PYL6* and *PYL12*) showed more plant growth while maintaining other traits (Miao et al. 2018). Similarly, *TaDREB2* and *TaDREB3* in wheat protoplasts were edited by CRISPR with almost 70% efficiency, demonstrating increased drought tolerance compared to wild type (Kim et al. 2018). *SAPK2*, one of the MAPK family members in rice and *SIMAPK3* in tomato were mutated by CRISPR/Cas9 which leads to increased tolerance against drought and salt stress (Lou et al. 2017; Wang et al. 2017a).

CBF genes impart tolerance to cold stress in plants, however it is a multi-membered gene family, for example 12 members in the case of rice and 3 in Arabidopsis. CRISPR/Cas9 was deployed to generate *cbf1cbf2* double mutant and *cbf1cpf2cpf3* triple mutant to study the significance of individual CBFs in cold stress tolerance (Jia et al. 2016). Regulation of stomatal density and stomatal index is an important trait for water use efficiency. CRISPR/Cpf1 was used to edit one of the regulators of stomatal density, *OsEPFL1*, which resulted in an improved stomatal patterning in rice under stress conditions (Yin et al. 2019). *SlNPR1* is

the ortholog of Arabidopsis *NPR1,* which regulates both abiotic and biotic resistance. The role of *SlNPR1* in drought has been found by editing through CRISPR and found that *slnpr1* mutant shows increased sensitivity to drought coupled with higher stomatal aperture and electrolyte leakage (Li et al. 2019b). Despite the above evidence were showing the potential of gene editing in generating stress tolerant crop plants, many stress-associated genes are difficult to be identified. Recently, in this context, targeting of structural, regulatory abiotic stress resistance genes and their cis-regulatory sequences through CRISPR/Cas was reported as one of the promising approaches for generating stress resilience crops (Zafar et al. 2019). Compiled information of a few CRISPR/Cas9-mediated stress resistance crops are furnished in Table 12.1. Readers are requested to go through recent reviews mentioned in this chapter for extensive examples on this aspect.

12.3.3 Defending Against Biotic Stressors

Crop yield reduction is fetched due to multiple biotic stress imposed by bacteria, fungi, viruses and nematodes. Transgenic plants expressing disease resistance genes, displayed enhanced tolerance against few pathogens, but this has also resulted in an upsurge of new strains of pathogens. To alleviate this problem, understanding of genes involved in plant-pathogen interaction is necessary. For example, S genes are the group of disease-causing genes in the plants. One of members is *OsERF922*, an ethylene responsive gene, whose knock out by CRISPR caused reduced blast infestation. Similarly, targeted editing of *OsSWEET13* gave rise to bacterial blight resistance in rice (Wang et al. 2016; Zhou et al. 2015). Targeted editing of effector binding elements in the promoter of *CsLOB1* in citrus increased disease resistance against *Xanthomonas citri* (Peng et al. 2017). Multiple gene editing of three homoeologs of *EDR1* in wheat, conferred resistance towards powdery mildew infection. Similarly, editing of *mlo* (mild resistance locus) alleles in Arabidopsis, wheat and barley resulted in resistance against *Blumeria graminins f.sp.tritici* (Wang et al. 2014). Similarly, viral diseases also cause huge yield losses unless they are controlled genetically. CRISPR techniques mutate the viral genome of pathogenic viruses in addition to controlling viral incidence in plants. For instance, FnCas9 driven by viral promoters, provided the viral resistance against TMV (tobacco mosaic virus) and CMV (cucumber mosaic virus) diseases (Zhang et al. 2017b, 2018b). Eukaryotic translation initiation factor *eIF4E* is the host factor essential for viral replication and mutation of this gene caused viral resistance in cucumbers (Chandrasekaran et al. 2016). Likewise, *eIF4G* is the negative regulator of viral resistance against rice tungro virus (RTV), and was knocked down by CRISPR to enhance disease resistance (Macovei et al. 2018). Wheat dwarf virus (WDV) is another serious threat in wheat and barley causing huge yield loss. Since there is a lack of natural resistance sources so far, CRISPR edited the conserved target site that has been discovered through mapping of WDV genome with PAM sequence and thus resulted in resistance to WDV (Kis et al. 2019). As we have seen before in abiotic stress

Table 12.1 A compiled information on stress resistance for major crops developed through CRISPR/Cas9

Crop	Target gene	Biotic stress	Abiotic stress	GE result	References
Rice	*eIF4G*	Tungro virus	–	Knock out	Macovei et al. (2018)
	OsERF922	Blast fungus	–	Knock out	F. Wang et al. (2016)
	OsSweet13	Bacterial leaf blight	–	Knock out	Zhou et al. (2015)
	OsNAC041	–	Salt	Knock out	Bo et al. (2019)
	OsOTS1	–	Salt	Knock out	Sadanandom et al. (2019)
	OsRR22	–	Salt	Knock out	A. Zhang et al. (2019)
	OsNAC14	–	Drought	Knock out	Shim et al. (2018)
	OsSAPK1&2	–	Salt	Knock out	Lou et al. (2018)
	OsAnn3	–	Cold	Knock out	Shen et al. (2017)
	SAPK2	–	Drought & Salt	Knock out	Lou et al. (2017)
	MPK2, PDS, BADH2	–	Multiple stress	Knock out	L. Wang et al. (2017)
Wheat	*EDR1*	Powdery Mildew	–	Knock out	Y. Zhang et al. (2017b)
	TaDREB2&3	–	Drought	Knock out	Kim et al. (2018)
Maize	*ARGOS8*	–	Drought	Knock out	Shi et al. (2017)
Cotton	*Gh14-3-3d*	Wilt	–	Knock in	Z. Zhang et al. (2018c)
Grapes	*VvWRKY52*	*Botrytis*	–	Knock out	X. Wang et al. (2018b)
Tomato	*SlJAZ2*	Bacterial Speck	–	Knock out	Ortigosa et al. (2019)
	CP & Rep sequences	Leaf curl virus	–	Knock out	Tashkandi et al. (2018)
	SlMlo1	Powdery mildew	–	Knock out	Nekrasov et al. (2017)
	SlNPR1	–	Drought	Knock out	Li et al. (2019)
	SlCBF1	–	Cold	Knock out	Li et al. (2018)

(continued)

Table 12.1 (continued)

Crop	Target gene	Biotic stress	Abiotic stress	GE result	References
	SlMAPK3	–	Drought	Knock out	L. Wang et al. (2017)

Source Razaaq et al. (2019). International Journal on Molecular sciences

section, there is a lot more to be explored for developing disease resistance with respect to CRISPR-based disease management strategies. In particular, the common hub of regulatory genes involved in the invasion by different pathogens have to be explored and could be edited through CRISPR technologies. Readers are requested to go through the review, published recently, for the updated information on crop-wise details for genome editing (Manghwar et al. 2019). Hereby, the compiled information of CRISPR/Cas9- mediated stress resistance crops are furnished in Table 12.1.

12.3.4 Other Key Applications of CRISPR

Mutant libraries are generated with the purpose of analysing gene functions systematically, through whole-genome mutagenesis or forward genetic screening. Also, the creation of mutant library is feasible for whole-genome sequenced plants such as rice, Arabidopsis, wheat. Since CRISPR/Cas system is a powerful tool for generating mutants, it was exploited to generate whole-genome mutant library. In the recent past, 12,802 genes were selected based on their high expression in rice shoot tissue and corresponding 25,604 sgRNAs were generated to create large-scale CRISPR mutant library (Meng et al. 2017). Similarly, Lu and his team generated 90,000 transgenic plants by targeting 34,234 genes in rice (Lu et al. 2017). In tomato, mutant library was generated by pooling sgRNA collections and large-scale mutant screening has also been carried out. From these mutants, alleles of leucine-rich repeat XII genes were identified which played a role in plant immunity (Jacobs et al. 2017). When these mutants were grown for screening, phenotype and genotype correlation was easily facilitated through sgRNAs (Bao et al. 2019). This evidence shows that CRISPR mutant libraries might play a crucial role in crop improvement in the coming decades.

Large deletion, translocation and inversion at genomic scale are some of the promising outputs of the CRISPR/Cas system. It drives the breeding approaches forward in terms of removal of the entire gene cluster, and establishing new linkages by translocation, transferring desirable traits from wild types by inversions of chromosomes (Puchta 2017). In rice, large-scale deletions of 245 kb and inversion of 300 bp by CRISPR/Cas9 has been demonstrated but their heritability has not been investigated so far (Zhou et al. 2014; Liang et al. 2016). Like deletion, targeted insertion of genomic fragments is quite possible through the HR-mediated pathway by providing a DNA repair template. In tomato, Geminivirus replicon was used to

supply both the repair template and CRISPR/Cas9-driven ANT1 construct- which led to dark purple coloration (Čermák et al. 2015). Biolistic transformation was also used to supply large fragments as the donor template. For instance, in maize, biolistic delivery of repair template targets *ALS2* and edited by HR resulted in resistance to chlorsulfuron (Svitashev et al. 2015). Alternatively, intron mediated gene replacement has been demonstrated for gene insertion through CRISPR/Cas9. In this method, sgRNA was designed to target two introns spanning the exon. Along with donor DNA template, Cas9 was supplied for targeted replacement (Li et al. 2016a).

Domestication is the process of generating modern crops through breeding by the introgression of desirable traits. Through this process, introgression of wild type traits into elite takes a long time. However, the process of domestication has been carried out only for major crops like rice, wheat and maize leaving out other important food crops. Now, CRISPR/Cas9 enabled the domestication process within a short time and produced elite crops from wild types with great agronomic traits through targeted mutations. For example, pennycress an important oil seed crop is improved with shorter life cycle, cold tolerance and increased oil production. Through CRISPR, its undesirable traits like seed dormancy (*DOG1*) and glucosinolate accumulation (*HAG1 and GTR2*) were modified to generate domesticated pennycress. Similarly, wild relative of tomato, ground cherry was modified by genome editing technologies to produce higher yield and larger fruits (Sedbrook et al. 2014; Lemmon et al. 2018).

Transgene free editing is one of the key concerns in creating CRISPR-edited crops for the public. Integration of Cas9 or vector backbone sequences makes it difficult to generate foreign DNA free plants. To circumvent this issue, DNA vectors and RNP complex approaches were executed (Li et al. 2019a). In the first approach, CRISPR/Cas9 delivery through *Agrobacterium*-mediated transformation allows the integration and editing of the construct at different chromosomes. Through segregation, transgene free edited plants were created in the next generation (Li et al. 2016a, 2017b; Wang et al. 2014). Also, transient transformation of CRISPR/Cas9 mediated by *Agrobacterium* have yielded transgene free edited plants in T0 generation in wheat and tobacco (Zhang et al. 2016; Chen et al. 2018). In recent past, TKC (transgenic killer CRISPR/Cas9) system was developed to self-eliminate transgenes through its suicide components like CMS2 and Barnase which kills the transgenes in the pollen and embryo, respectively. This led to transgene free T0 generations in rice (Rodríguez-Leal et al. 2017). In the second approach, Cas9 protein and sgRNA are assembled in vitro into an RNP complex and delivered to plants by biolistic transformation. After editing, RNP was degraded since it is devoid of foreign DNA, which eventually leads to transgene free plants. This technique was already deployed in rice, lettuce, tobacco and Arabidopsis. For instance, in maize, *als2* mutants were obtained through HR by the co-delivery of ALS2: RNP complex with single stranded DNA template by particle bombardment method (Svitashev et al. 2016).

12.4 Regulatory Aspects on CRISPR Plants

The potential of genome editing in crops has been getting enormous attention and growing tremendously. However, the discrepancies exist for gene edited (GE) crops in terms of its safety and adaptation. The regulatory networks for the approval of GE crops has some bottlenecks which hampers their development as well as marketability. Despite the commercialization of GMO crops since last several years, improper understanding and mistrust are still prevailing with public due to strict regulations by the Government. Basically, the major difference between genome editing crops with GMO, should be clarified to the regulatory bodies. Also, it is imperative to educate them about the recent progress in CRISPR techniques and improved delivery systems, which do not necessitate the insertion of foreign DNA into the crop plants. Besides, CRISPR-edited crops lead to rapid crop improvement, free of transgenes and produce genetic variability in a better way than transgenic and mutation breeding. In fact, the cost for producing genome-edited crops is around 30 US dollars which is surprisingly lesser compared to quarter of million US dollars for producing transgenic plants. In addition to saving money, it reduces laborious process, year-round field trails which altogether helpful for removing the barriers existing over GM crops (Baltes et al. 2015; Visser et al. 2001; Ledford 2015).

The assessment and acceptance of CRISPR-edited crops for its direct or long-term effect as food and as a feed varies from country to country. It affects the trading of genome-edited (GE) crops between two countries with different legislative procedures. So, it is important to consider the type of GE techniques used and their delivery methods (Jansing et al. 2019b). Till now, there is no international regulatory framework for GE crops worldwide. However, USA and Europe are the major stakeholders, having opposite legislation policies. GE crops have not been grouped under the category of GMO, and so granted permission to be developed and marketed in the US whereas in Europe, GE is included under GMO category with a notion that it could result in an unknown risk because of the genomic manipulation (Fears and Ter Meulen 2017). The consortium of research organizations in Europe, EPSO (The European Plant Science Organisation) have expressed their unpleasant opinion for the ban on genome editing. European government should focus on product-based research rather than method-based restrictions in scientific discoveries (Schulman et al. 2020). Unlike Europe, USDA in 2016, had ruled out the regulations for GE mushroom and corn and got them in the US markets. Along with USA, Canada, Brazil, Argentina, Chile, Australia have joined to draft new regulatory framework by discarding certain rules in order to absolve GE crops from GMO. Those new regulations are mainly concerned about developing GE crops free of transgenes, devoid of pest incidences and modification of traits (Razzaq et al. 2019). Interestingly, in Canada, PNT (plant with novel traits) regulations are followed for the crops attaining specific traits through traditional breeding, mutagenesis, genetic engineering and genome editing technologies (Smyth 2017). In Argentina, the legislation is cleared for any crops free of transgenes (null segregants) through drafting flexible assessment for developing GE crops. Despite transgenic techniques were used, the final

product should be free of foreign genes for the approval in Argentina (Whelan and Lema 2015).

In China, CRISPR is being used extensively in the field of agriculture and medicine and it has been featured as CRISPR revolution in China by Science journal recently (Cohen 2019), indicating that China is being the leading player of GE crop research. Also, an inventory analysis revealed that China is dominating among the 2000 patent applications dealt with CRISPR and USA is marginally ahead of China. Food security is considered as the major reason behind China's major interest towards CRISPR in academics and industries, in order to meet out its ever-expanding population. Also, 'China daily' paper reported that despite China showing great interest in GE crops, it lacks clear regulatory policies that arrest the development of CRISPR crops in the laboratory itself. An initiative was established recently in which twelve Chinese academicians were signed in the draft to look after the strict legislation on GE crops, which will probably increase the quality of outcome and competitiveness on GE technologies. Moreover, the recent findings followed by the accusation of 'CRISPR babies' in China have led to make strict regulations on genome editing, not only in mammalian research but also in the field of agriculture and pharmaceuticals.

In India, the regulation and bio safety evaluation of GMOs are under strict scrutiny and that framework has been formulated earlier along with other countries. The rules are governed under Environment Protection Act, 1996, by Ministry of Environment, Forest and Climate Change. From time to time, the policies are being upgraded depending on the new findings of the research (Choudhary et al. 2014; Warrier and Pande 2016).

Despite genome editing being more precise than natural mutagenesis, this technique is far from acceptance in many countries. This could be additionally due to lack of technical conveyance to the public and thought process of considering GE as a GMO. Although, CRISPR crops are accepted in few countries, co-ordination in the legal policy should be standardized at the global level to increase the marketability of CRISPR crops in future.

12.5 Conclusion with Perspectives

The range of applicability of CRIPSR has been recently burgeoning mainly because of its low cost, technical rapidity, easy execution and precised editing at genome level. The new developments such as prime editing, base editing, multiplexing, epigenome modifications will further help in increasing the horizons of CRISPR applications in various crops. In plant systems, CRISPR is being used effectively for the past five years to develop new varieties, with improved agronomic traits and resistance traits against biotic and abiotic stresses (Razzaq et al. 2019). However, those edited plants are confined in laboratory environments. Probably, in near future, more plants could

be bred with CRISPR technologies and get ready for the markets. Despite its significant progress, it has many rooms for improvements and technical challenges. HR-mediated genetic editing is one such challenge ahead for successful CRISPR application. Also, the effective delivery of donor template through Geminivirus vectors, utilization of NHEJ inhibitors and HR enhancers, have led to some output but remarkable progress is still under debate. Another priority of improvement lies in its efficient delivery method because vast number of major crop plants are lacking proper transformation methods and/or difficult to transform. Carbon nanotubes, silica nanoparticles and layered double hydroxides are some newly suggested delivery methods for precise GE. Also, the other concern is about the improvement in targeted editing rather than off-targeting. With the advent of improved CRISPR vectors, these issues could be overcome easily by the research community in near future. Despite excellent nuclease functionality, CRISPR efficiency basically depends on the target gene sequence, cell type and epigenetic state of the chromosome. Eventually, genome editing should be combined with other functional genomic approaches like next generation sequencing, synthetic and systems biology in order to reap the complete benefits of technologies for crop improvement in the current era of ever evolving climatic conditions.

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Chapter 13
CRISPR-Cas12a (Cpf1) and Its Role in Plant Genome Editing

Jonathan Windham, Shailendra Sharma, Manoj Kumar Kashyap, and Sachin Rustgi

Abstract Since the discovery of DNA, a large number of advancements were made in the field of molecular biology, which has improved our ability to decipher the Pandora's box of decoded plant and animal genomes. This knowledge can be used to benefit humanity by making precise genetic alterations in plant and animal genomes. One such technology is CRISPR (Clustered Regularly Interspaced Short Palindromic Repeat), which is increasingly being used for genome editing in plants and animals. The CRISPR technology is being used not only in elucidating the gene function but also to precisely alter gene function in humans and agricultural plants. CRISPR systems evolved naturally in bacteria to defend against viruses. CRISPR-associated protein (Cas) 9, Cas12 (including Cpf1), Cas13, and Cas14 are variants of this novel bacterial immune system, which were repurposed for genome or RNA editing. The purpose of this chapter is to provide a brief introduction to CRISPR technology, precisely CRISPR-Cas12a, and its implications in plant genome editing.

Keywords CRISPR · Genome editing · Cas12a (Cpf1) · Genetic engineering · Guide RNA · Crop improvement · Plant breeding

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G. Tang et al. (eds.), *RNA-Based Technologies for Functional Genomics in Plants*,
Concepts and Strategies in Plant Sciences, https://doi.org/10.1007/978-3-030-64994-4_13

Abbreviations

Cas	CRISPR-associated protein
Cpf1	CRISPR from *Prevotella* and *Francisella* 1
CRISPR	Clustered regularly interspaced short palindromic repeats
crRNA	CRISPR RNA
DSBs	Double-strand breaks
gRNA	Guide RNA
HDR	Homology-directed repair
INTEGRATE	Insert transposable elements by guide RNA-assisted targeting
NHEJ	Non-homologous end joining
PAM	Protospacer adjacent motif
TALEN	Transcription activator-like effector nuclease
tracrRNA	Trans-activating crRNA
ZFN	Zinc-finger nuclease

13.1 Introduction

CRISPR (Clustered Regularly Interspaced Short Palindromic Repeat)-Cas (CRISPR-associated) system is a revolutionary tool, contemporarily used for targeted genome editing. On the contrary, tools such as zinc-finger nuclease (ZFN) and transcription activator-like effector nuclease (TALEN) are phasing out due to the technical difficulties associated with their designs, which require protein restructuring with each target (Langner et al. 2018). On the other hand, CRISPR is relatively easy to design (Chandrasegaran and Carroll 2016); therefore, the scientific community is shifting toward CRISPR technology, and widely accepted it as the standard genome editing tool.

Francisco Mojica coined the term "CRISPR" in 1990s (Mojica et al. 2005). However, it was not until 2012, when Jennifer Doudna and Emmanuelle Charpentier's group has demonstrated the use of programmable RNA-guided genome editing in an *in vitro* system (Jinek et al. 2012). Subsequently, in 2013, the application of the CRISPR-Cas system was demonstrated in editing mammalian (Cong et al. 2013; Mali et al. 2013) and plant genomes (Li et al. 2013; Nekrasov et al. 2013; Shan et al. 2013). CRISPR enables researchers to edit genomes in various ways, including gene silencing, activation, suppression, and introduction, to name a few among different CRISPR applications (cf. Zhang et al. 2019; Lemmon et al. 2018; Rodríguez-Leal et al. 2017). These CRISPR applications have facilitated researchers in altering plant and animal genomes in various ways to feed multiple needs of humankind (Chen et al. 2019; Zaidi et al. 2019; Hickey et al. 2019; Bailey-Serres et al. 2019; Eshed and Lippman 2019).

The CRISPR locus consists of an array of small repeats, which are the hallmark of a bacterial immune system that formed the basis for CRISPR-Cas genome editing technology. The CRISPR technology is an umbrella term used for different variants and orthologues of Cas9 and Cas12a, which were put to a variety of uses (cf. Zhang et al. 2019). Both CRISPR-Cas9 and -Cas12a depend on the PAM and guide RNA for target site recognition. CRISPR technology enables not only modification of genes in living cells but also many *in vitro* applications, such as genomic DNA complexity reduction or targeted sequence enrichment, to facilitate the sequencing of complex genomes (Paul Doran, iGenomX, personal communication) or detection of DNA/RNA molecules at attomolar level (cf. Wang et al. 2019) and various small molecules at nanomolar level (Liang et al. 2019; Dai et al. 2019). The use of CRISPR technology was also demonstrated in recording information in and retrieving information from the cells (Shipman et al. 2017). The new uses of the CRISPR technology are regularly being discovered, which is overwhelmingly benefiting both the agricultural and medical sciences. However, this immense power to modify genomes come with greater ethical and moral responsibilities (Brokowski and Adli 2019). Therefore, the regulations to restrict the unethical use of these technologies are also being parallelly sought.

13.2 Where Does CRISPR Come from

Spanish researcher, Francisco Mojica of the University of Alicante, first reported the CRISPR-Cas system in Archaea and later in bacteria and hypothesized its role in providing immunity from invading bacteriophages and plasmids (Chen and Doudna 2017). CRISPR locus possesses short (~20–50 bp) direct repeats interspersed by sequences derived from foreign invading DNA dubbed “protospacer.” The system serves as a recorder that helps the cell to detect and destroy the returning invaders. In 2007, Mojica’s theory was experimentally validated by a team of researchers led by Philippe Horvath (cf. Barrangou et al. 2007). Later Doudna and Charpentier repurposed the bacterial CRISPR-Cas9 system for genome editing in prokaryotes and demonstrated its application in an *in vitro* system (Doudna and Charpentier 2014). Whereas, Feng Zhang and colleagues demonstrated its first application in genome editing of eukaryotes in an *in vivo* system (Joung et al. 2017).

13.3 Discovery and Characterization of the CRISPR-Cas12a System

The continued quest for new CRISPR variants and availability of the massive amount of sequencing data from bacteria and archaea lead to the discovery of a putative class 2 nuclease, dubbed Cpf1 (Zetsche et al. 2015). Later, the gene was annotated in

several genomes and classified as a novel, type V CRISPR system. Similar to Cas9, Cas12a contains a RuvC-like endonuclease domain, but unlike Cas9, it lacks the HNH endonuclease domain (Zhang et al. 2019). It suggests that Cas12a functions differently from Cas9. Since Cas12a loci are commonly present in different bacterial species, it was hypothesized that Cas12a might represent a functional CRISPR nuclease which could be deployed in genome editing. Upon testing, the Cas12a displayed cleavage activity in mammalian cells (Zetsche et al. 2015). After searching for cellular RNAs essential for Cas12a functioning, it was found that Cas12a interacts with a crRNA of 42–44 nucleotides (nt) to induce cleavage in the target DNA sequences. Further exportations revealed that Cas12a has a preference for a 5′-TTTV (V represents A, G, and C) PAM sequence for target recognition (Li B et al. 2018). The size of the crRNA used by Cas12a is about the same size as Cas9, but unlike Cas9, in Cas12a crRNA, the direct repeat precedes the spacer. Typically, Cas12a crRNA is composed of a 20-nt direct repeat (also known as a 5′ handle) and a 23-nt spacer (guide segment) (Fig. 13.1) (Li B et al. 2018). The direct repeat in crRNA adopts a pseudoknot structure, which contains Watson–Crick base pairs. The spacer in crRNA is complementary to the target DNA sequence, and the seed region located at the first eight nucleotides of the spacer has a critical role in the target specificity (Li B et al. 2018).

There are several advantages that Cas12a offers as a tool for genome editing (Li B et al. 2018; Zaidi et al. 2017): (1) The PAM requirement allows Cas12a to target T-rich regions of the genome, which are more evenly distributed in genomes. (2) Cas12a only requires a short crRNA (~42 nt), making it easy to synthesize, multiplex, and engineer. (3) In addition to DNA nuclease activity, Cas12a also possesses RNase activity, which allows the processing of a CRISPR array hence making it amenable for use in multigene editing. (4) Cas12a generates DSBs with staggered ends distal from

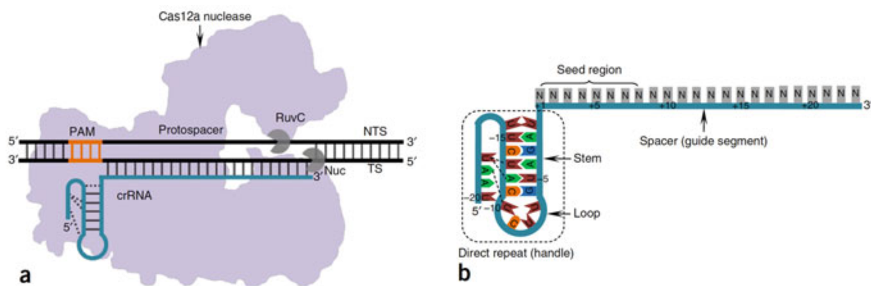

Fig. 13.1 Diagrammatic representation of the CRISPR–Cas12a system. **a** AsCas12a-mediated site-specific DNA double-stranded breaks. CRISPR–Cas12a is a two-component system comprised of a non-sequence specific endonuclease (purple) and a single crRNA (steel blue). PAM = protospacer-adjacent motif (5′-TTTV-3′; orange); RuvC and Nuc (gray) = the Cas12a domains involved in cleaving the non-target and target DNA strands, respectively; crRNA = CRISPR RNA; NTS = non-target strand; and TS, = target strand. **b** Schematic illustration of the AsCas12a crRNA. crRNA consists of a direct repeat (5′ handle) and a spacer (guide segment). This figure is modified from Li B et al. (2018)

the PAM site, which allows continuous cleavage of DNA and may promote NHEJ (non-homologous end joining)-based gene knockouts or HDR (homology-directed repair)-mediated gene insertion (due to the cohesive DNA ends). (5) Cas12a is also considered more specific than wild-type Cas9, as demonstrated in several biological systems. Given these beneficial attributes, the three Cas12a nucleases one each from *Acidaminococcus* spp. BV3L6 (AsCpf1), *Lachnospiraceae bacterium* ND2006 (LbCpf1), and *Francisella novicida* U112 (FnCpf1) were used to edit rice, maize, Arabidopsis, tobacco, tomato, soybean, cotton, and citrus genomes (Table 13.1) (cf. Zhang et al. 2019).

Similar to Cas9, Cas12a also contains a recognition (REC) and a nuclease (NUC) lobe (Fig. 13.1). However, the introduction of mutations at the catalytic residues in the RuvC domain at the NUC lobe of Cas12a was shown to abolish its cleavage activity at both DNA strands at the target site (Zhang et al. 2019). Therefore, no Cas12a nickase is currently available. However, multiple versions of catalytically dead Cas12a (dCas12a) have been engineered, such as dAsCas12a, dLb-Cas12a, and DNase-dead Cas12a (ddCas12a), and repurposed for different applications (cf. Zhang et al. 2019). Furthermore, to broaden the target ranges of Cas12a, mutations were also introduced in different domains of the enzyme to generate RR and RVR variants. Similar mutations are now introduced into the rice-codon-optimized LbCas12a, FnCas12a, and AsCas12a (Schindele and Puchta 2020; Zhang et al. 2019). Additionally, Tang et al. (2017) attached the dead Cas12a with the SRDX repression domain and demonstrated its potential for transcriptional repression in plants, which makes this system an attractive tool for regulating plant gene expression in addition to genome editing.

13.4 Other CRISPR Systems

The computational and functional analyses have uncovered a large number of CRISPR systems, which were broadly classified based on the number of enzymes required for processing of pre-crRNA and interference. The CRISPR system is grouped as class 2 if a single multifunctional protein accomplishes both pre-crRNA processing and interference. Otherwise, it is grouped as a class 1 system (Zhang et al. 2019; Langner et al. 2018; Chen and Doudna 2017). Each class is further divided into multiple types, for instance, type I, III, and IV belong to class 1, with Cas3, Cas10, and Csf1 as their signature proteins, while type II (Cas9), type V (Cas12a–e, Cas12g–I, and Cas14a–c) and type VI (Cas13a–d) belong to class 2 (Zhang et al. 2019; Langner et al. 2018). Each type of CRISPR system can be further grouped into subtypes based on the organization of operon and Cas proteins at the CRISPR loci. Shmakov et al. (2017) have recently changed the nomenclature of type V and VI enzymes as follows—Cas12a (formerly Cpf1) and Cas12b (formerly C2c1), Cas12d (formerly CasY), and Cas12c (formerly CasX), and Cas13a (formerly C2c2) to keep the consistency.

Table 13.1 Summary of various Cas12a transformation studies in various plants

Crop	Explant	Target gene	Transformation method	Cas12a species	Mutation frequency	Reference
Arabidopsis	Whole seedlings	*AtPDS3*	*Agrobacterium*	AsCas12a, LbCas12a	See reference	Bernabé-Orts et al. (2019)
Arabidopsis	Protoplasts	*AtGL2*, *AtTT4*	Polyethylene Glycol (PEG)	LbCas12a	0–35%, 0–15%	Malzahn et al. (2019)
Arabidopsis	Whole seedlings	*ALS* (AT3G48560)	*Agrobacterium*	LbCas12a	6.1%	Wolter and Puchta (2019)
Peanut (*Arachis hypogaea*)	Shoot apex	*Ara h1*, *Ara h2*, *Ara h3*, *Ara h6*	*Agrobacterium*	LbCas12a	N/A	Alam T, Hoover G, Gandhi N, Rustgi S, unpublished results
Chlamydomonas reinhardtii	Cells	*FKB12*, *CpFTSY*, *CpSRP4*, *PHT7*	Electroporation	LbCas12a	0.5–16%	Ferenczi et al. (2017)
Duncan grapefruit (*Citrus paradise*)	Epicotyl	*CsLOB1*	*Agrobacterium*	LbCas12a	15–55%	Jia et al. (2019)
Cucumber (*Cucumis* sp.)	Protoplasts	*Csa1G085890*, *Csa6G292430*, *Csa5G623470*	PEG	LbCas12a	N/A	Uslu T, Ozdemir BS, Rustgi S, unpublished results

(continued)

Table 13.1 (continued)

Crop	Explant	Target gene	Transformation method	Cas12a species	Mutation frequency	Reference
Soybean (*Glycine max*)	Protoplasts	*FAD2-1A*, *FAD2-1B*	PEG	AsCas12a, LbCas12a	0–11.7% for FAD2-1A and 0–9.1% for FAD2-1B (LbCpf1), 0–1.6% for FAD2-1A and 0–0.6% for FAD2-1B (AsCpf1)	Kim et al. (2017)
Nicotiana benthamiana	Leaf discs	*XT1*	*Agrobacterium*	AsCas12a, LbCas12a	0%, 54%	Bernabé-Orts et al. (2019)
Wild tobacco (*Nicotiana attenuate*)	Protoplasts	*AOC* (ALLEN OXIDE CYCLASE)	PEG	AsCas12a, LbCas12a	100%	Kim et al. (2017)
Nicotiana benthamiana (transgenic GFP-expressing)	Leaves	GFP locus	*Agrobacterium*	AsCas12a, LbCas12a	N/A	Ferenczi et al. (2017)
Tobacco (*Nicotiana tabacum*)	Leaf discs	*NtPDS*, *NtSTF1*	*Agrobacterium*	FnCas12a	12.5–65.2% (crNtPDS-1), 4.3–50% (crNtPDS-2), 28.6–68.2% (crNtSTF1-4)	Endo et al. (2016)
Tobacco (*Nicotiana tabacum*)	Protoplasts	*PDS*	*Agrobacterium*	FnCas12a	See reference	Hsu et al. (2019)

(continued)

Table 13.1 (continued)

Crop	Explant	Target gene	Transformation method	Cas12a species	Mutation frequency	Reference
Physcomitrella Patens	Protoplasts	*HDS19*, *HDS21*, *HDS22*, *HSFA1*, *HSFA2*, and *HSFB4*	Heat shock	LbCas12a	26.5–100%	Pu et al. (2019)
Peach (*Prunus persica*)	Seeds	*PpTFL1*	Biolistic	LbCas12a	N/A	Windham J, Gasic K, Rustgi S, unpublished results
Chickasaw plum (*Prunus angustifolia*)	Seeds	*PaTFL1*	Biolistic	LbCas12a	N/A	Windham J, Gasic K, Rustgi S, unpublished results
Tomato (*Solanum lycopersicum*)	Cotyledons	MYB12	*Agrobacterium*	AsCas12a, LbCas12a	See reference	Bernabé-Orts et al. (2019)
Wheat (*Triticum* sp.)	Calli	*Gli1*, *Gli2*, *Glu3*	Biolistic	LbCas12a	N/A	Kashyap et al. (2019); Rustgi et al. (2019)
Corn (*Zea mays*)	Immature zygotic embryos	*gl2* (*glossy2*)	*Agrobacterium*	LbCas12a	0–60%	Lee et al. (2019)

AsCas12a derived from *Acidaminococcus* sp. | **FnCas12a** derived from *Francisella novicida* | **LbCas12a** derived from *Lachnospiraceae bacterium*

13.5 How CRISPR Functions

The direct repeat and protospacer array at the CRISPR locus transcribes into a non-coding precursor CRISPR RNA (pre-crRNAs), which gets processed by a single Cas protein or a pack of proteins to mature crRNA (Chen and Doudna 2017). The crRNA consists of a single direct repeat (~42 nt in Cas9 and 20 nt in Cas12a) and a spacer (17–20 nt in Cas9 and 22/23 nt in Cas12a). This crRNA either directly as in the case of Cas12a or first by binding to the trans-activating crRNA (tracrRNA, ~65 nt in size) as in Cas9, forms a ribonucleoprotein complex with Cas enzyme and guides it to the target sequence in the genome (Zaidi et al. 2017; Swarts and Jinek 2018). Hence this RNA is dubbed as guide RNA. As mentioned earlier for Cas12a, it consists exclusively of crRNA with a 20-nt direct repeat and 22/23-nt spacer. The term sgRNA (single guide RNA, synthetic guide RNA, or short guide RNA) refers to a synthetic alternative for Cas9 protein that combines both the crRNA and tracrRNA elements into a single RNA molecule. Once the target DNA is found, a process that depends on the identification of the PAM sequence, the nuclease Cas9/Cas12a, induces a double-stranded DNA break (DSB) at the target site. The DSB is then repaired by the cell's inherent repair machinery, either via NHEJ or HDR (Chen and Doudna 2017).

13.6 Comparison of CRISPR-Cas9 and CRISPR-Cas12a Systems

There are several endonucleases which could be employed with the CRISPR system (Koonin et al. 2017; Murugan et al. 2017). Among different nucleases, Cas9 dominates the current literature. Of the newer endonucleases discovered, Cas12a (Cpf1) has emerged as an attractive alternative to Cas9. There are some fundamental differences between CRISPR-Cas9 and CRISPR-Cas12a. These differences have been summarized in Table 13.2. In contrast to Cas9, where the endonuclease needs to forms a complex with two small RNAs (crRNA and tracrRNA) for inducing breaks in DNA, Cas12a requires only a single RNA (crRNA). The size of Cas12a is relatively smaller than Cas9, which makes its delivery easier into cells/tissues. Another major difference between Cas9 and Cas12a is that Cas9 produces "blunt ends," whereas Cas12a induces "staggered ends" (Fig. 13.2). Cas12a cuts distal to the PAM site, whereas Cas9 induces breaks proximal to the PAM site. Like Cas9, the Cas12a-gRNA complex first recognizes the PAM sequence and then induces DSBs at the target site; therefore, the targets are selected based on the presence of an appropriate, adjacent PAM sequence (TTTV). Both Cas9 and Cas12a have a preference for different PAM sequences which could be advantageous in targeting different parts of the genome with contrasting GC contents with the two nucleases. Cas12a generates larger deletions than Cas9 (Tang et al. 2017), which bestows Cas12a the more mutagenic power. Furthermore, unlike Cas9, Cas12a does not utilize tracrRNA

Table 13.2 Difference between Cas9 and Cas12a (Cpf1)

	CRISPR-Cas9	CRISPR-Cas12a
Organism	*Streptococcus pyogenes*, *Streptococcus thermophilus*, *Staphylococcus aureus*, *Neisseria meningitidis*, *Campylobacter jejuni* *Francisella novicida* *Treponema denticola* *Staphylococcus canis* *Brevibacillus laterosporus* *Staphylococcus macacae*	*Francisella novicida*, *Acidaminococcu* spp., *Lachnospiraceae bacterium*, *Prevotella* spp.
Subtype	Subtypes II-A, II-B, and II-C	Subtypes V-A (Cpf1) and V-B (C2c1)
Type	Type II CRISPR-Cas systems	Type V CRISPR-Cas systems
Size	~1,000–1,600 aa	~1,100–1,300 aa
Spacer length	18–24 nt	18–25 nt
Structure of gRNA	Consists of two RNA molecules (crRNA and tracrRNA) or a synthetic molecule, dubbed "sgRNA"	Consists of one RNA molecule (crRNA)
Pre-crRNA processing	No	Yes
tracrRNA	Yes	No
Total guide length	~100 nt (sgRNA)	42–44 nt
PAM	• 3′ G-rich PAM, e.g., 3′-NGG (SpCas9), 3′-NNGRRT (SaCas9), 3′-NNNNGATT (NmCas9) • Cutting site is proximal to PAM	• 5′, T-rich PAM, e.g., 5′-TTTV (LbCas12a, AsCas12a) • Cutting site is distal to PAM
Cutting mechanism	Blunt-ended dsDNA break	dsDNA staggered end break (sticky ends)

and RNase III to process pre-crRNA. Pre-crRNA processing is accomplished by the Cas12a nuclease itself (Swarts and Jinek 2018; Zetsche et al. 2015).

The self-processing of pre-crRNAs by the Cas12a system is an important feature because it allows for simplified multiplex gene editing (Zetsche et al. 2017). Cas9 can be used for multiplex gene editing, however, doing so typically requires individual expression cassettes for each gRNA (Kabadi et al. 2014; Jinek et al. 2012). Other complicated construct designs, shown in Fig. 13.3 and as discussed in Zhang et al. (2019), Minkenberg et al. (2017), Najera et al. (2019), Nissim et al. (2014), Sakuma et al. (2014), Tsai et al. (2014), and Xie et al. (2015) were also tested. Multiple, individual expression cassettes increase the size of the constructs used and thus hinder transfection. This physical limitation reduces the number of sites that Cas9 can target simultaneously. Alternatively, in the case of Cas12a, multiple crRNAs of

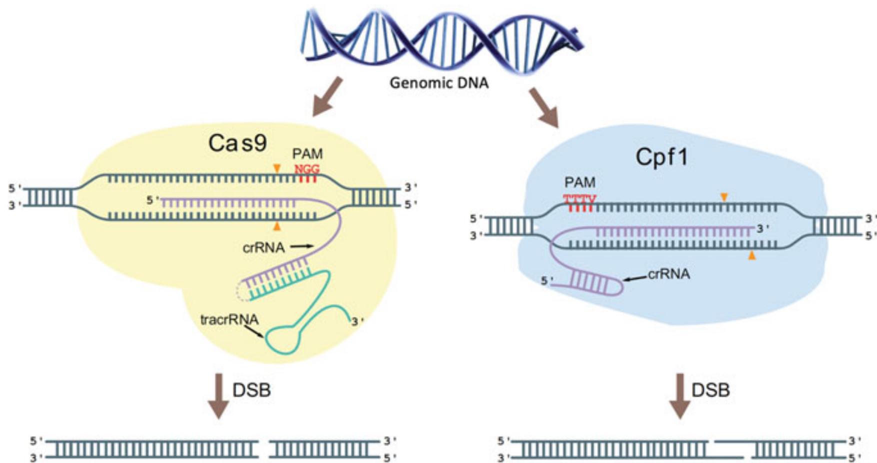

Fig. 13.2 Diagrammatic representation of the difference in cleavage pattern of CRISPR-Cas9 and CRISPR-Cas12a (Cpf1). Notice a G-rich site (NGG) acts as a protospacer adjacent motif (PAM) in case of CRISPR-Cas9, whereas a T-rich (TTTV) site on the 5′—end of the target region in case of Cas12a, differences in need of two vs. one RNA molecule for endonuclease targeting to the site of interest, type of double-stranded break (DSB) produced blunt vs. staggered, and the distance of cut site from the PAM. This figure is modified from Vanegas et al. (2020)

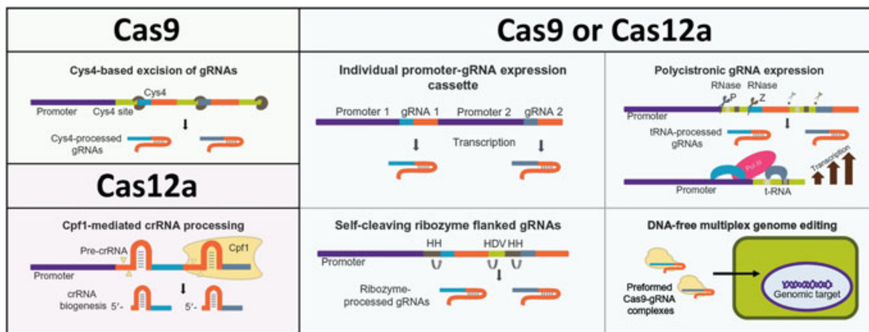

Fig. 13.3 Multiplex guide (g)RNA expression strategies. Multiplexed gRNA can be expressed using Cys4 and drosha-mediated gRNA–short hairpin (sh)RNA system (top left panel); multiple gRNA expression cassettes, gRNA flanked on either side by ribozymes (center panel), polycistronic transfer RNA (tRNA)–gRNA expression cassette, or ribonucleoprotein (RNP) complex (right panel); CRISPR array (bottom left panel). This figure is modified from Minkenberg et al. (2017). HH = hammerhead ribozyme; HDV = hepatitis delta virus ribozyme

42–44 nt each can be packed in a single plasmid with a recent report citing as many as 25 crRNAs carried on a single plasmid (Campa et al. 2019). The simplicity and targeting capacity of Cas12a make it the preferable tool for multiplex gene editing.

13.7 Applications of Cas12a (Cpf1) in Plant Genome Editing

Although, gene editing through CRISPR-Cas12a in plants is still in its infancy, this system is proving to be a valuable addition to the plant genome editing toolbox. As Cas12a is further optimized, there is no doubt that it will be utilized in staple crops, fruits and vegetables, and ornamentals. As such, genome editing tools like Cas12a are highly sought after to facilitate the task of incorporating multiple desired traits into a single genotype. Below we briefly discussed some examples where CRISPR-Cas12a has been applied for genome editing in plants like cotton, maize, and rice. Other Cas12a-based studies in plants have been summarized in Table 13.1.

13.7.1 Cotton

The allotetraploid *Gossypium hirsutum* (upland cotton) is the most widely planted species of the genus, accounting for more than 90% of production worldwide (Zhang et al. 2015). The fibers found in cotton are composed almost entirely of cellulose. These fibers are actually elongated extensions of single-cells found on the seed epidermis (Haigler et al. 2012). Fiber yield, while important, is not alone significant enough to guarantee the commercial success of a variety. A variety must also exhibit high fiber quality. Factors such as fiber length, fiber strength, fineness (fiber diameter), and fiber color (Lacape et al. 2005; Zhang et al. 2009) are critical to the market performance of a variety. While most reports of gene editing in cotton have been in reference to Cas9 (Manghwar et al. 2019), the use of Cas12a has been also reported. One such report was an experiment undertaken to examine the efficiency of Cas12a in allotetraploid cotton, targeting the gene *GhCLA* (*cloroplastos alterados* or "altered chloroplasts") that is vital to chloroplasts biogenesis. Disruption of *CLA* results in an albino phenotype. Results indicated a high level of gene editing, with an extent of mutation ranging from 1% to 94.12% (Li B et al. 2019). These authors also reported that the Cas12a system seems to introduce large deletions (3–28 bp) more often than smaller insertions/deletions or substitutions. Lastly, the group reported that in more than 50% of their sample specimens, edits were created in both allotetraploid subgenomes, simultaneously. Given these results, Cas12a may prove to be a more attractive gene editing system in cotton compared to Cas9.

13.7.2 Maize (Corn) and Sorghum

Maize (*Zea mays*) is a globally important staple crop, accounting for nearly 15% of the world's protein intake and 20% of the world's caloric intake (Nuss and Tanumihardjo 2010). Maize is also a versatile crop with a wide range of applications such as allowing

for the production of sweeteners, starches, cooking oil, alcohols, and biofuels like ethanol (Ranum et al. 2014). The maize genome, consisting of 2.3 gigabases across 10 chromosomes, was sequenced in 2009 and revealed that nearly 85% of the genome consisted of transposable elements (Schnable et al. 2009). Despite difficulties in tissue culture and transformation, maize is the crop with the greatest number of transgenic commercial varieties (Yadava et al. 2017). The significance of the crop, the availability of a draft genome, and the extent of the transgenic work already accomplished in maize make it an attractive choice for gene editing.

Attempts have been made to utilize the Cas12a system in maize with suboptimal results (Lee et al. 2019). Authors reported that while Cas12a did indeed generate mutations in the targeted gene (*glossy2*), rates were lower than those obtained by using Cas9. Cas12a was also able to generate biallelic mutations in maize, however, these rates were also lower in comparison with those of Cas9. Mutations generated by both systems were heritable. The low targeting efficiency of Cas12a in this study could possibly be attributed to temperature sensitivity. Malzahn et al. (2019) reported that the Cas12a nucleases appear to be optimal between 28 °C and 29 °C, with mutation frequencies in maize as high as 100% using LbCas12a at 28 °C (Malzahn et al. 2019). These temperature ranges are not ideal for every plant and efforts have been made to engineer variants of LbCas12a that are efficient at lower temperatures (Schindele and Puchta 2020). These results are promising; however, further optimization is needed.

At the time of this publication, work utilizing the Cas12a system in sorghum was in the preliminary stages. Despite the advantages that Cas12a may offer, the majority of reports regarding gene editing in sorghum employ Cas9 (Char and Yang 2019). This may simply be due to the fact that Cas12a is the more recent discovery (Zetsche et al. 2015) compared to Cas9. Publications examining the capabilities of Cas12a in sorghum will surely come.

13.7.3 Rice

Rice is one of the most widely consumed cereal grains in the world. Nearly 480 million tons of rice are produced annually, with China and India accounting for almost half of that production (Muthayya et al. 2014). Of the roughly 23 species (Vaughan et al. 2003) in the genus, only two are grown for consumption: *Oryza sativa* and *Oryza glaberrima* (Muthayya et al. 2014). Rice was the first crop to have its genome sequenced and in 2006, the International Rice Genome Project published their results (Jackson 2016). This enormous accomplishment opened up new insight into the genetic diversity and domestication of rice while setting the stage for future crop improvement. Fortunately for breeders and researchers, *O. sativa* is known for its ease of transformation (Hirochika et al. 2004; Kyozuka and Shimamoto 1991; Sasaki et al. 2002), making it a popular choice for Cas12a editing (Table 13.3).

Table 13.3 Summary of various Cas12a transformation studies in rice

Crop	Explant	Target gene	Transformation method	Cas12a species	Mutation frequency	Reference
O. sativa subsp. *japonica* cv. “Nipponbare”	Protoplasts	*OsPDS*, *OsDEP1*, *OsROC5*	Polyethylene Glycol (PEG)	AsCas12a, LbCas12a	0.6–10%, 15–25%	Tang et al. (2017)
O. sativa subsp. *japonica* cv. “Nipponbare”	Calli	*OsPDS*, *OsDEP1*, *OsROC5*	*Agrobacterium*	LbCas12a	100%	Tang et al. (2017)
O. sativa subsp. *japonica* cv. “Nipponbare”	Protoplasts	*OsDEP1*, *OsROC5*	Polyethylene Glycol (PEG)	AsCas12a, FnCas12a, LbCas12a	Temperature-dependent, see reference	Malzahn et al. (2019)
O. sativa subsp. *japonica* cv. “Nipponbare”	Calli	*OsPDS*, *OsBEL*	*Agrobacterium*	LbCas12a	crRNA length-dependent, see reference	Xu et al. (2017)
O. sativa subsp. *japonica* cv. “Nipponbare”	Calli	*OsDL*, *OsALS*	*Agrobacterium*	FnCas12a	8.3–25% (crOsDL-1), > 60% (crOsDl-2), 15% (crOsALS-1), and > 60% (crOsALS-2)	Endo et al. (2016)
O. sativa subsp. *japonica* cv. ‘Zhonghua 11’	Calli	*OsALS*	Biolistic	LbCas12a	N/A	Li S et al. (2018)
O. sativa subsp. *japonica* cv. “Nipponbare”	Calli	*OsALS*	Biolistic	LbCas12a	70.8%	Li S et al. (2020)
O. sativa cv. “Kitaake”	Calli	*CAO1*	Biolistic	FnCas12a	8%	Begemann et al. (2017)

(continued)

Table 13.3 (continued)

Crop	Explant	Target gene	Transformation method	Cas12a species	Mutation frequency	Reference
O. sativa subsp. *japonica*	Calli	*OsEPSPS*, *OsBEL*, *OsPDS*	*Agrobacterium*	FnCas12a, LbCas12a	See reference	Wang et al. (2017)
O. sativa subsp. *Japonica*	Calli	*OsRLK*-798, *OsRLK*-799, *OsRLK*-802, *OsRLK*-803	*Agrobacterium*	FnCas12a	43.8–75%	Wang et al. (2017)
O. sativa subsp. *Japonica*	Calli	*OsBEL*-230, *OsBEL*-240, *OsBEL*-250, *OsBEL*-260	*Agrobacterium*	LbCas12a	40–60%	Wang et al. (2017)
O. sativa subsp. *indica* cv. "IR64"	Immature embryos	*OsEPFL9*	*Agrobacterium*	LbCas12a	> 14%	Yin et al. (2017)
O. sativa subsp. *japonica* cv. "Nipponbare"	5-day-old mature seed derived rice embryos	*OsPDS*	Biolistic RNP/DNA co-delivery	AsCas12a, LbCas12a	0.0% (AsCas12a), 11.7% (LbCas12a)	Banakar et al. (2020)
O. sativa	Protoplasts	*OsMPK2* and *OsMPK5*	PEG–$CaCl_2$ solution	FnCas12a LbCas12a	9–32% (LbCas12a), 2–9% (FnCas12a)	Ding et al. (2018)
O. sativa subsp. *japonica* cv. "Nipponbare"	Protoplasts	*OsPDS*, *OsDEP1*, *OsROC5*, and *OsEPFL9*	PEG	FnCas12a LbCas12a	Up to 90% (FnCas12a), up to 100% (LbCas12a)	Zhong et al. (2018)
O. sativa subsp. *japonica* cv. "Nipponbare"	N/A	*Narrow Leaf1* (*NAL1*), *LIGULELESS1* (*LG1*)	*Agrobacterium*	LbCas12a	5%	Hu et al. (2017)
O. sativa subsp. *japonica* cv. "Nipponbare"	Protoplast	*OsEPFL9*, *OsGS3*	PEG	AaCas12b AacCas12b	10%, 5%	Ming et al. (2020)

AsCas12a derived from *Acidaminococcus* sp. | **FnCas12a** derived from *Francisella novicida* | **LbCas12a** derived from *Lachnospiraceae bacterium* | **AaCas12b** derived from *Alicyclobacillus acidoterrestris* | **AacCas12b** derived from *Alicyclobacillus acidiphilus*

13.8 Looking Forward

The CRISPR-Cas systems are adept at eliciting responses from DNA repair mechanisms within the cell. The non-homologous end joining (NHEJ) repair pathway is one repair option, however, it is typically error-prone and thus results in random indel sequences at the target locus. Homology-directed repair (HDR) is a viable option to introduce an indel of a known sequence, however, this pathway is challenging and often met with very low efficiency (Rozov et al. 2019). The desire to effectively insert any DNA sequence of interest into a target genome, combined with the intellectual property (IP) issues regarding commercial use of the CRISPR-Cas technology, has driven researchers to seek alternative tools capable of high-efficient knock-ins. The newly discovered INTEGRATE (INsert Transposable Elements by Guide RNA-Assisted TargEting) technology has the incredible potential to fill this role (Klompe et al. 2019). This system offers the opportunity for site-specific DNA integration while avoiding the need for double-stranded breaks at a target locus. The INTEGRATE system is the latest addition to the CRISPR toolbox and is expected to complement existing CRISPR systems including Cas3, Cas9, Cas12, Cas13, Cas14, and their variants (Pickar-Oliver and Gersbach 2019).

While the CRISPR-Cas system is most notable for its precise genome editing capabilities, efforts have been made to utilize the system to serve other purposes. Tang et al. (2017) reported success in using modified AsCas12a and LbCas12a to induce transcriptional repression in *Arabidopsis*. The researchers mutated AsCas12a and LbCas12a in order to deactivate the nuclease domains of each and then fused them to three copies of the SRDX transcriptional repressor. While LbCas12a is typically considered to be the more efficient endonuclease compared to AsCas12a or FnCas12a (Bernabé-Orts et al. 2019; Langner et al. 2018; Schindele and Puchta 2020), it was demonstrated that the deactivated-AsCas12a was the more efficient transcriptional repressor, possibly due to tighter DNA binding. It has also been reported that Cas12a can be utilized in biosensing applications (Li Y et al. 2019), which may greatly impact areas such as plant pathology, phytoremediation, and ecology.

There is still much to be explored regarding the CRISPR-Cas12a system. As this technology becomes more widely available and more cost-effective we can expect to see further creative applications in plants, not only in the area of crop improvement, but also for the creation of ornamental novelties. Under the new SECURE Rule (85 FR 29790-29838, Docket No. APHIS-2018-0034), the United States Department of Agriculture has chosen not to regulate CRISPR-edited crops (USDA 2018; Waltz 2016), which will hopefully incentivize researchers and speed improved varieties to market. Ideally, engagement and transparency with the public should be encouraged to foster understanding of this technology and trust, respectively.

Whatever the application, it's clear that CRISPR is a powerful tool that has far-reaching ramifications. The new possibilities unlocked by this technology should be thoroughly explored, expanded upon, and even celebrated as we enter the new age of genetic engineering.

Acknowledgements This work was supported by the SC Peach Council, National Peanut Board, and NIFA Hatch/Multi-state grant (S009). JW and SR would like to thank the Chair of the Department of Plant and Environmental Sciences and the Director of the Pee Dee Research and Education Center, Clemson University, for providing facilities. MKK would like to thank the management of the Amity University Haryana, India for providing resources and facilities, and the Teachers Association for Research Excellence (TARE) fellowship from the Science and Engineering Board (SERB), Government of India, New Delhi. Thanks are also due to the Department of Biotechnology (DBT), Govt of India, for providing funds in the form of research projects to SS.

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

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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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G. Tang et al. (eds.), *RNA-Based Technologies for Functional Genomics in Plants*,
Concepts and Strategies in Plant Sciences, https://doi.org/10.1007/978-3-030-64994-4_14

Keywords Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR) · Type VI CRISPR/Cas system · Cas13 · crRNA · RNA cleavage · RNA editing in plants

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.

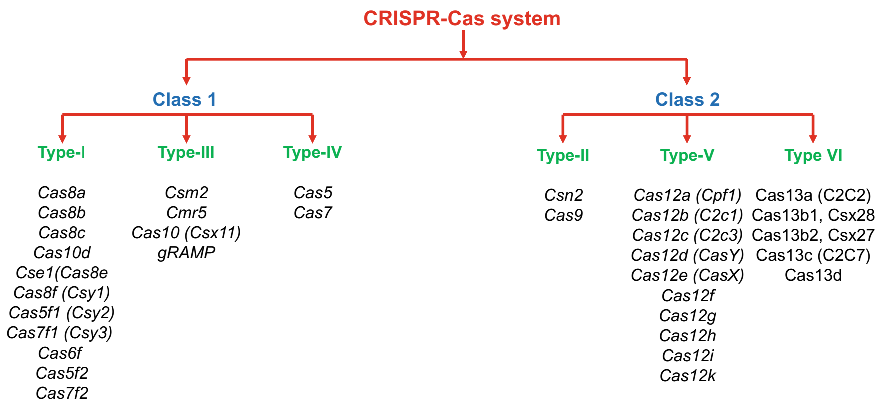

Fig. 14.1 The Classes, types, and subtypes of CRISPR/Cas systems: CRISPR-Cas system is divided into two classes: Class 1 and Class 2 on basis of Cas proteins. Class 1 has a set of effector complexes while class 2 contains a single protein. Each class consists of 3 types: Class 1 system contains type 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).

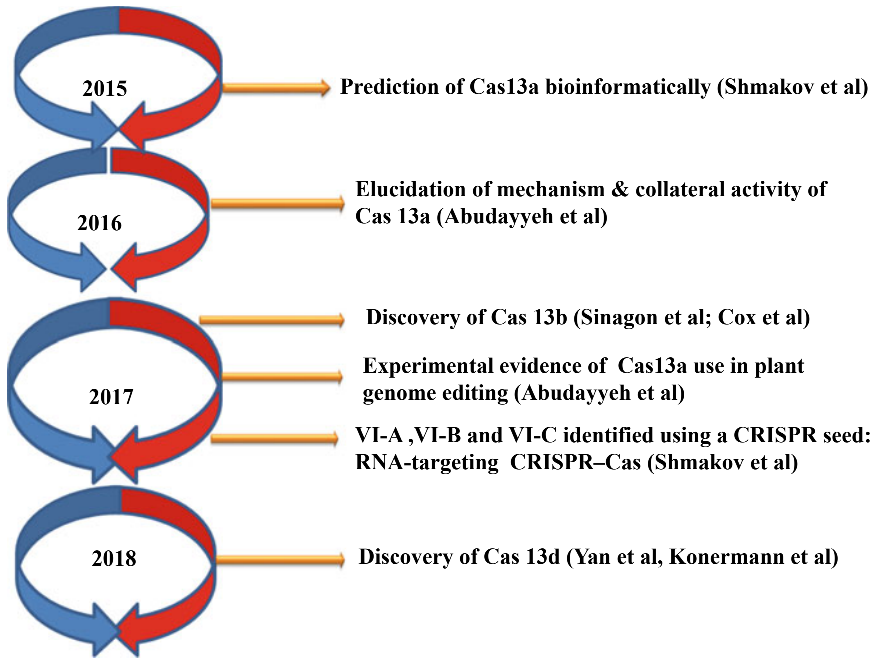

Fig. 14.2 Historical timeline of the discovery of type VI CRISPR/Cas system

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).

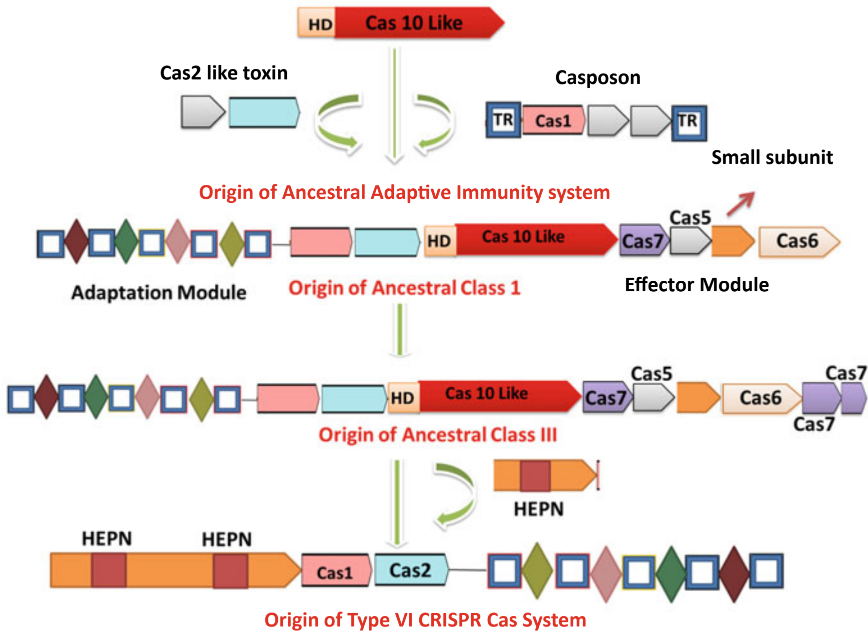

Fig. 14.3 Evolutionary Scenario for Type VI CRISPR/Cas System: Evolutionary scenario of type VI CRISPR-Cas systems initiated with the origin of ancestral adaptive immunity system initiated by fusion of Cas10-like gene with casposon and Cas2-like toxin. Origin of ancestral class I occurred by fission 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).

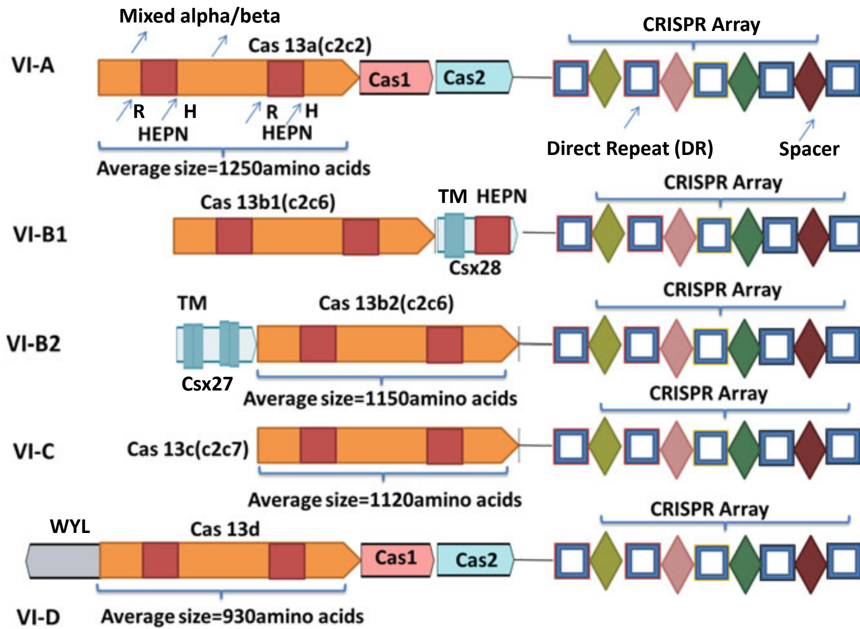

Fig. 14.4 Structural Variations of Type VI CRISPR/Cas systems: VI-A *Leptotrichia shahii;* VI-B1: *Prevotella buccae*; VI-B2: *Bergeyella zoohelcum*; VI-C: *Fusobacterium perfoetens*; VI-D: *Ruminococcus bicirculans*. The principal organization domains of CRISPR-Cas locus are adaptation module/an operon 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

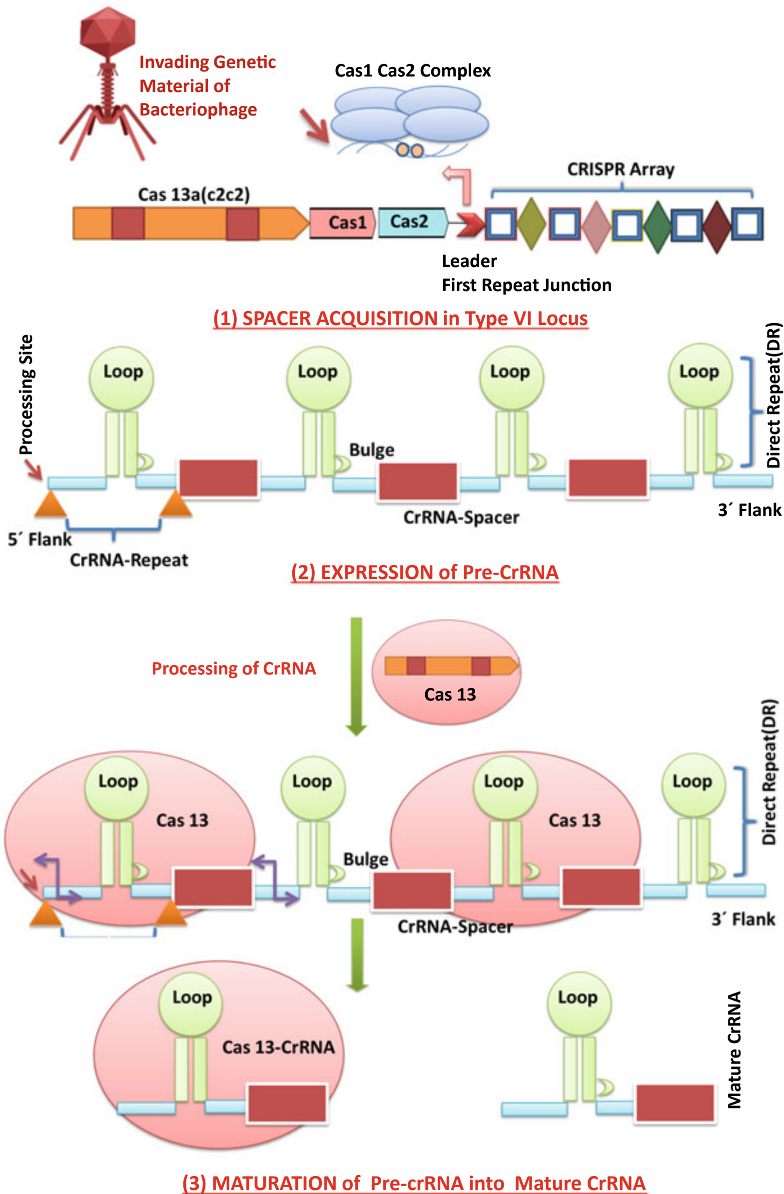

Fig. 14.5 Mechanism of Type VI CRISPR-Cas Systems: Type VI CRISPR/Cas functions in four stages: (1) Adaptation or spacer acquisition in type VI locus wherein short fragments of invading RNA are acquired by Cas1–Cas2 and integrated at leader sequence as new spacers by adaptation. (2) Expression or transcription 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

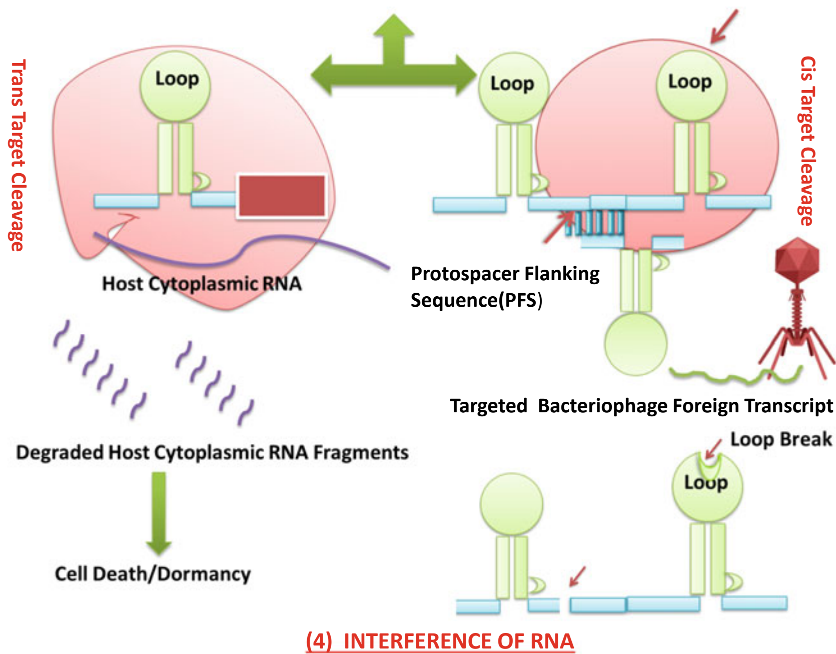

Fig. 14.5 (continued)

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

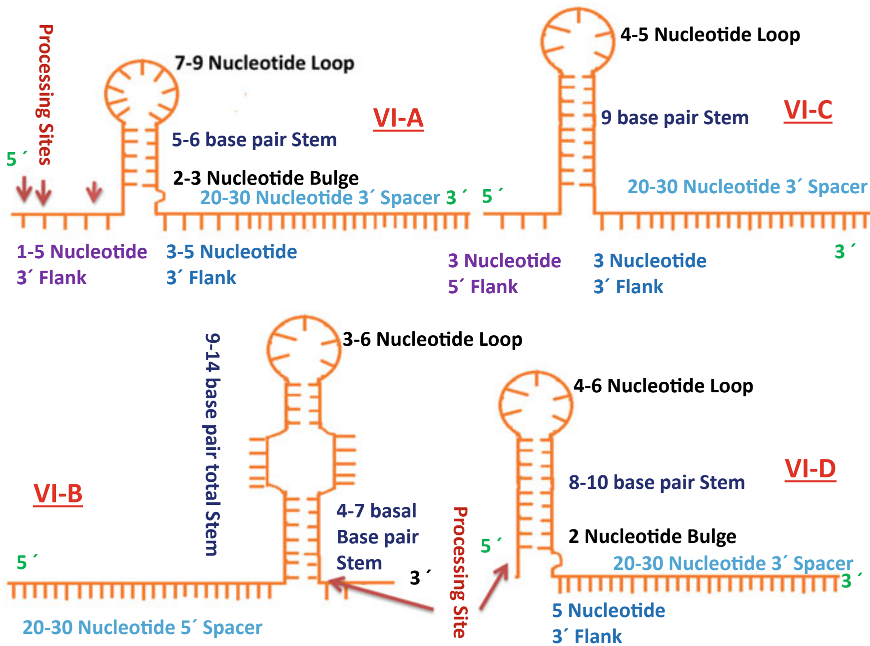

Fig. 14.6 Diverse Emphasized Characteristics of crRNAs in four variants of Type VI: Schematic representation of main parallels and variance in features of VI-A, VI-B, VI-C, VI-D crRNAs. Spacer length varies in VI-A, VI-C, VI-D from 20 to 30 nt 3′ whereas VI-B has 20–30 nt 5′ spacer on expression in *E. 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.

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Chapter 15
Mutagenomics for Functional Analysis of Plant Genome using CRISPR Library Screen

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Abstract Genetic engineering will help to accomplish global food demand by increasing crop productivity, nutritional quality, and making the plants tolerant toward abiotic and biotic stresses. The clustered regulatory interspaced short palindromic repeats (CRISPR/Cas9) is an advanced genetic engineering technology, which is employed in many plant species and has several advantages (precise, faster, and multiple site gene editing) over the conventional mutagenesis methods. CRISPR/Cas9 tool enables programmable genome editing, transcriptome regulation, and epigenetic editing. CRISPR-mediated genome editing has successfully been employed in several crops (monocots and dicots) such as Arabidopsis (*Arabidopsis thaliana*), rice (*Oryza sativa*), tomato (*Solanum lycopersicum*), maize (*Zea mays*), and *Brassica* spp, among others by targeted mutagenesis. Different high-throughput CRISPR screening methods were also used in plant functional genomics research. This chapter discusses about the targeted mutagenesis in plants by using CRISPR technology and various screening methods for CRISPR library.

Keywords CRISPR/Cas9 · CRISPR library · Genetic engineering · Mutagenesis · Screening methods

15.1 Introduction

Increasing crop productivity is a major goal of current agriculture which is severely affected due to depleting tillable land, water resource and adverse impact of global climate change. Novel technological intervention in plant breeding will help to sustain increasing demands for food and nutrition. Mutation breeding or mutagenesis is the key to introduce new traits in existing crop varieties or breed new crops with improved traits (Oladosu et al. 2016).

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G. Tang et al. (eds.), *RNA-Based Technologies for Functional Genomics in Plants*, Concepts and Strategies in Plant Sciences, https://doi.org/10.1007/978-3-030-64994-4_15

Mutagenesis involves techniques that enables researchers to introduce heritable changes in the genome to enhance/repress gene(s) of interest. CRISPR-mediated genome editing opened a new possibility for mutagenesis in plants. These tools have enabled researchers to modify the plant genome for introducing both transient and heritable genotypic changes. Many molecular genetics techniques have been used before for crop mutagenesis such as T-DNA insertion, meganucleases, zinc finger nucleases (ZFNs), and transcription activator-like effector nucleases (TALENs). Most of the tools mentioned above have shown double-stranded break (DSB) of DNA, which is followed by conventional cell DNA repair machinery through non-homologous end joining (NHEJ) or homology-directed repair (HDR). NHEJ and HDR can efficiently introduce insertions or deletions i.e., indels of various space and length in the open reading frame (ORF), transacting site, promoter binding site or enhancer-binding site (Zaboikin et al. 2017). With these (meganucleases, ZFNs and TALENs) nuclease-based genome-editing system, chances of getting desired mutant increases significantly. However, each of the above tools have their limitations for instance, ZFNs or TALENs are extremely tedious to work and shows high off-target mutations. The new genome-editing tools i.e., CRISPR associated protein 9 nuclease (CRISPR/Cas9) have overcome almost all the drawbacks of the conventional nuclease-based editing tools. CRISPR/Cas9 is present in prokaryotes and archaea as their immune system (Horvath and Barrangou 2010). CRISPR protects the lower organisms from foreign nucleic acid invasion such as viruses (Barrangou et al. 2007) or plasmids by cleaving or modifying it. CRISPR associated protein 9 nuclease (CRISPR/Cas9) edits dsDNA via its RNA-guided Cas9 endonuclease, by mutating the dsDNA target.

CRISPR-Cas9 system is classified into three major types; type I, II, and III and many are unclassified (Makarova et al. 2011). CRISPR/Cas9 belongs to type II classification, which contains an endonuclease Cas9 and RNA complex (crRNA and tracrRNA) (Jinek et al. 2012). This RNA complex in the CRISPR enables Cas9 for target-based editing of DNA (Deltcheva et al. 2011). The crRNA (CRISPR RNA) contains variable targeting sequence which can be engineered complementing the target DNA. However, the non-variable parts of tracrRNA (trans-activating crRNA) are of long stretch of bases that form stem-loop structure (Li 2014). The protospacer adjacent motif (PAM) consists of 2–6 bp recognition site that changes with change in host and CRISPR system. Engineered sgRNA (single guide RNA; custom-designed short crRNA sequence along with scaffold tracrRNA sequence) corresponding to the crRNA in the RNA complex directs the Cas9 to the specific target site of DNA by using complementary base pairing. The specified target DNA must occur immediately after 5′ PAM from 5′ NGG. Different Cas9 orthologues may differ in the requirement of PAM motifs and sgRNA scaffold structures to provide efficient binding and creating DSBs, followed by a conserved repair mechanism (Kleinstiver et al. 2015). Being very precise and efficient in its ability, CRISPR/Cas9 has provided the possibility of genome editing in all organism including bacteria, animals and plants.

15.2 CRISPR/Cas9-Mediated Targeted Mutagenesis in Plants

At present, the most commonly used techniques for genome editing are the ZFNs (zinc finger nucleases), TALENs (transcription activator-like effector nucleases) or the recently developed CRISPR/Cas9-mediated system. Although ZFNs and TALENs have been successfully employed for genome editing, each has its unique limitations, and their implementation in a plant is far from regular use. In contrast, the CRISPR provides many advantages such as designing, multiplexing, specificity; cost-effectiveness, and flexibility over the ZFNs- and TALENs-based approaches. Initially, successful application of plant genome editing (via CRISPR/Cas9-mediated) was reported in 2013 on *Oryza sativa*, *Arabidopsis thaliana,* and *Nicotiana benthamiana* plants (Li et al. 2013; Miao et al. 2013; Nekrasov et al. 2013; Shan et al. 2013; Arya et al. 2020). Plants have shown high efficiency for CRISPR/Cas9-mediated genome editing which greatly depends upon its target genome locus, sgRNA and Cas9. The availability of user-friendly computational tools for designing gRNAs or predicting off-targets of genome editing has made the wide adoption and applications of this technique. Therefore, the CRISPR/Cas9-based technology is getting wide attention from the researchers and its application has extended significantly in different areas of plant sciences (Abdelrahman et al. 2018; Bao et al. 2019; Kishi-Kaboshi et al. 2018; Langner et al. 2018; Najera et al. 2019; Schindele et al. 2018; Soda et al. 2018). Last few years, several crops such as rice (*O. sativa*), tomato (*S. lycopersicum*), *Arabidopsis (Arabidopsis thaliana),* rapeseed (*Brassica* spp.), tobacco (*Nicotiana tabacum*), soybean (*Gycine max*), maize (*Zea mays*), wheat (*Triticum aestivum*), barley (*Hordeum vulgare*), sorghum (*Sorghum bicolor*), potato (*Solanum tuberosum*), flax (*Linum usitatissimum*), false flax (*Camelina sativa*), cotton (*Gossypium hirsutum*), ipomoea (*Ipomoea purpurea*), cucumber (*Cucumis sativus*), lettuce (*Lactuca sativa*), grapes (*Vitis vinifera*), cassava (*Manihot esculenta*), alfalfa (*Medicago sativa*), apple (*Malus domestica*), orange (*Citrus sinensis*), carrot (*Daucus carota*), watermelon (*Citrullus lanatus*), salvia (*Salvia officinalis*), and cacao (*Theobroma cacao*) have been edited by using CRISPR (Table 15.1). Furthermore, CRISPR library is one of the important tools for rapid creation of mutant population. We describe here CRISPR library-based approach for mutagenesis in detail.

Recently, multiple functional studies successfully described various screening approaches such as large scale mutagenesis, transcriptional repression or activation through CRISPR/Cas9-mediated genome editing (Fig. 15.1). Therefore, the genome-wide CRISPR libraries would prove to be a robust and valuable tool for generating large scale mutant resources in plants for functional genomics analysis and agronomic trait improvement (Table 15.2).

Table 15.1 Year-wise genome editing in crops/plants by using CRISPR technology

Genome editing in crops/plants	Years	References
Rice *(Oryza sativa)* Wheat *(Triticum aestivum)* Maize *(Zea mays)*	2013	Xie and Yang (2013) Shan et al. (2013) Zhang et al. (2013)
Tomato *(Solanum lycopersicum)* Citrus *(Citrus sinensis)*	2014	Brooks et al. (2014) Jia and Wang (2014)
Potato *(Solanum tuberosum)* Chinese white poplar *(Populus tomentosa)* Soybean *(Glycine max)*	2015	Wang et al. (2015b) Fan et al. (2015) Sun et al. (2015)
Grapes *(Vitis vinifera)*	2016	Ren et al. (2016)
Watermelon *(Citrullus lanatus)* Cotton *(Gossypium hirsutum)* Cassava *(Manihot esculenta)* Ipomoea *(Ipomoea purpurea)* Barrel medic *(Medicago truncatula)*	2017	Tian et al. (2017) Gao et al. (2017) Odipio et al. (2017) Watanabe et al. (2017) Meng et al. (2017b)
Alfalfa *(Medicago sativa)* Carrot *(Daucus carota)* Cacao *(Theobroma cacao)* Salvia *(Salvia miltiorrhiza)* Lettuce *(Lactuca sativa)*	2018	Gao et al. (2018) Klimek-Chodacka et al. (2018) Fister et al. (2018) Zhou et al. (2018) Bertier et al. (2018)
Cowpea *(Vigna unguiculata)* Wild citrus *(Fortunella hindsii)* Cabbage *(Brassica oleracea)*	2019	Ji et al. (2019) Zhu et al. (2019) Ma et al. (2019)

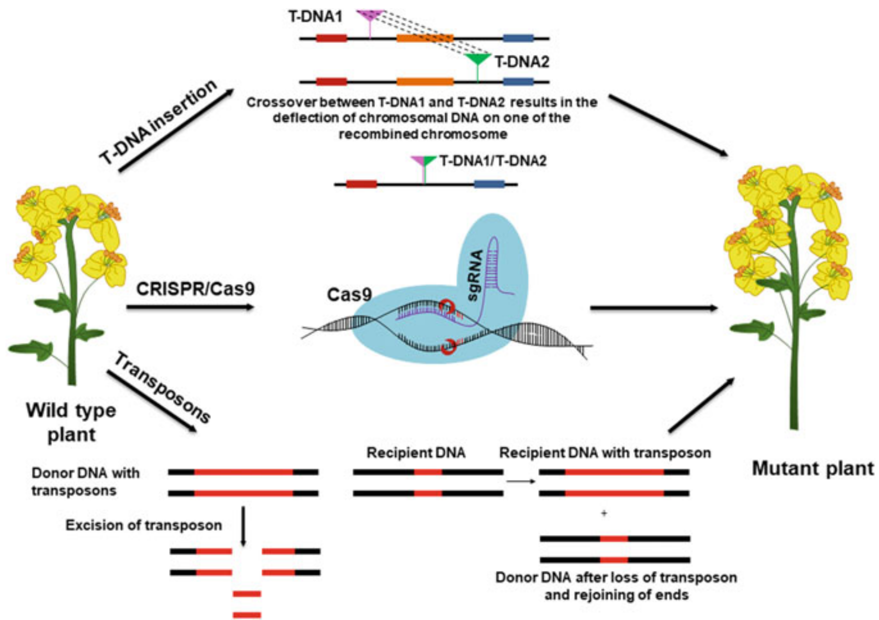

Fig. 15.1 Schematic representation of different molecular biology methods for mutagenesis (T-DNA insertion, CRISPR/Cas9 and Transposon) for the improvement of traits in plants. While T-DNA insertion and transposon-based mutagenesis process are random, CRISPR/Cas9-based editing is very precise and targeted

15.3 Construction of the CRISPR Library

Gene function validation has been aided by the ability to generate targeted gene knockouts or transcriptional suppression using the CRISPR/Cas9 system. CRISPR/Cas9 library can be constructed by ribonucleoprotein (RNP) assembly or cloning multiple gRNAs into the destination vector using various methods (Liang et al. 2017).

Designing of sgRNA: Various computational tools are available for, on-target (CRISPR activity) and off-target (specificity) scoring. For example, CRISPRdirect (http://crispr.dbcls.jp), SSFinder (https://code.google.com/p/ssfinder), CasFinder (http://arep.med.harvard.edu/CasFinder), CCTop (http://crispr.cos.uni-heidelberg.de), Cas-Designer (http://www.rgenome.net/cas-designer), E-CRISPR (http://www.e-crisp.org/E-CRISP/), CRISPRer (http://jstacs.de/index.php/CRISPRer), CRISPRTarget (http://bioanalysis.otago.ac.nz/CRISPRTarget), CRISPOR (http://crispor.tefor.net/crispor.cgi), CRISPRfinder (http://crispr.u-psud.fr/Server), CRISPRP (http://cbi.hzau.edu.cn/crispr), Cas-OFFinder (http://casoffinder.snu.ac.kr) and CasOT (https://omictools.com/casot-tool) have been developed to make CRISPR gRNA (Chuai et al. 2017) designing user-friendly and reliable. All these *in silico* gRNA design and off-target prediction tools have significantly facilitated the wide

Table 15.2 Improvements of plant traits by using target specific genes through CRISPR technique and their functional evaluation in plants

Plants	Target gene	Phenotypes	References
Brassica napus	*BnALC, IND*	Increased shatter resistance	Braatz et al. (2017), Zhai et al. (2019)
Brassica olerecea	*BolC.GA4.a*	Dwarf stature and reduced fruit dehiscence	Lawrenson et al. (2015)
Triticum aestivum	*Ms1*	Complete male sterility	Okada et al. (2019)
	EDR1, Taedr1, TaMLO, TaLpx-1	Improved resistance to powdery mildew disease caused by *Blumeria graminis* fungi	Wang et al. (2014, 2018), Zhang et al. (2017)
	TaGW2	Enhancement of protein content in grain and gluten strength. Increased grain size, weight, and resistance to head blight disease caused by *Fusarium Graminearum* fungus	Borrelli et al. (2018), Zhang et al. (2018b)
Solanum lycopersicum	*SP5G*	Early flowering and enhancing the compact determinate growth	Soyk et al. (2017)
	SlMLO	Improved resistance to powdery mildew disease caused by *Oidium neolycopersici* fungus	Nekrasov et al. (2017)
	SlGAD2, *SlGAD*, *GABA-TP1*, *GABA-TP2*, *GABA-TP3*, *CAT9*, *SSADH*	Increased content of c-Aminobutyric acid non-protein amino acid in leaves and fruits	Li et al. (2018b), Nonaka et al. (2017)
	SlMAPK3	Improved tolerance to drought and heat stress	Yu et al. (2019)
	CCD8	Resistance to the parasitic weed *Phelipanche aegyptiaca*	Bari et al. (2019)

(continued)

Table 15.2 (continued)

Plants	Target gene	Phenotypes	References
	SHR	Elongated zones and stunted meristematic with short hairy roots in plants	Ron et al. (2014)
	SlORRM4, lncRNA1459	Fruit ripening delaying, inhibition of respiratory rate, ethylene and carotenoid biosynthesis pathway	Li et al. (2018a), Yang et al. (2017)
	SlIAA9	Altered leaf morphology, seedless fruits and increased plant growth rate	Ueta et al. (2017)
	SlPMR1, SlPMR2, SlPMR3, SlPMR4, SlPMR5, SlPMR6	Resistance against the pathogen *Oidium neolycopersici*, which causes powdery mildew disease	Seifi et al. (2014)
Oryza sativa	*TMS5*	Thermo-sensitive genic male sterility	Zhou et al. (2016)
	Gn1a, *DEP1*, *GS3*, *IPA1*	Increased grain number and size with dense erect panicles	Li et al. (2016)
	OsCCD7	High-tillering with dwarf plants	Butt et al. (2018)
	Waxy	Decreased amylose content and an enhanced glutinosity	Zhang et al. (2018a)
	OsNramp5	Reduced Cd content in grains	Tang et al. (2017)
	Oshkt1, nced5-1, nced5-2	Sensitive to salt stress	Huang et al. (2019), Wang et al. (2015a)
	Osmdh1, Osrr9/Osrr10	Improved tolerance to salt stress	Nan et al. (2020), Wang et al. (2019d)
	OsPRP1	Induced cold sensitivity	Nawaz et al. (2019)
	OsPDS	Enhancement of nutritional content	Li et al. (2017)

(continued)

Table 15.2 (continued)

Plants	Target gene	Phenotypes	References
	OsMPK5	Enhanced resistance against rice disease	Xie and Yang (2013)
	OsSWEET13	Improved resistance against bacterial blight disease caused by *Xanthomonas oryzae*	Kim et al. (2019)
	OsEPSPS, *OsMYB5*, *OsPMS3*	Mutant plants showed enhanced drought tolerance and decreased rate of cell survivals	Zhang et al. (2014)
	OsPPa6	Lowered the photosynthetic rate.	Wang et al. (2019a)
Arabidopsis thaliana	*ICE1*	Enhanced freezing tolerance	Ye et al. (2019)
	AREB1	Improved drought tolerance	Roca Paixao et al. (2019)
	tps5	Abscisic acid ABA sensitive during seed germination and effects during stomatal closure	Tian et al. (2019)
	aitr2,aitr5, aitr6 aitr256, dpa4, sod7	Increased seed size, and tolerance to drought and salt stresses	Chen et al. (2019)
	CBF1-3	Hypersensitive to cold stress	Robison et al. (2019)
	CLE9	Sensitive to drought stress	Zhang et al. (2019a)
	AtSIZ1	Reduced salt tolerance	Han et al. (2019)
	VP16-02-003, VP16-05-014	By improving the circadian cycle enhanced the growth	Augustijn et al. (2019)
	BRI1	Plants showed dwarf phenotype	Lynagh et al. (2018)
	CHLI1, CHLI2	Plants were albino and pale green	Mao et al. (2013)
Solanum tuberosum	*GBSS*	Increased amylose synthesis, starch level and amylopectin/amylose ratio	Andersson et al. (2017)

(continued)

Table 15.2 (continued)

Plants	Target gene	Phenotypes	References
	StALS1	Resistance to herbicide	Butler et al. (2015)
Arabis alpina	*AaSPL15*	Unable to flower after vernalization	Hyun et al. (2019)
Petunia hybrida	*PhACO1*	Reduced ethylene production and enhanced flower longevity	Xu et al. (2019)
Nicotiana benthamiana	*RDR6*	Sterile and abnormal flowers	Matsuo and Atsumi (2019)
Musa acuminata	*RAS-PDS*	Decreased the content of chlorophyll and carotenoid pigments	Kaur et al. (2018)
Gossypium hirsutum	*GhCLA1*	Plants showed albino phenotype	Wang et al. (2017)
	Gh14-3-3d	Resistance to *Verticillium dahlia* fungus causing cotton verticillium wilt	Zhang et al. (2018c)
Glycine max	*GmFEI2* *GmSHR*	Contributing in the efficiency of both exogenous and endogenous genes in soyabean hairy root.	Cai et al. (2015)
	GmFT2	Exhibiting late flowering under both long-day and short-day photoperiodism	Cai et al. (2018)
Citrus sinensis	*CsLOB1*, *EBEPthA4*	Resistance to Citrus canker disease caused by *Xanthomonas citri* subsp. *citri*	Peng et al. (2017)
Vitis vinifera	*IdnDH*	Stops the Tartaric acid biosynthesis and accumulation in suspension cells	Ren et al. (2016)
	MLO-7	Resistance against the powdery mildew disease caused by *Erysiphe necator* fungus	Pessina et al. (2016)

(continued)

Table 15.2 (continued)

Plants	Target gene	Phenotypes	References
	VvWRKY52	Enhanced resistance to bacterial and fungal pathogens like *Coniella diplodiella*, *Pseudomonas syringae* and *Golovinomyces cichoracearum*	Zhang et al. (2019b)
Lactuca sativa	*NCED4*	Rate of seed germination increases at high temperature	Bertier et al. (2018)
Theobroma cacao	*TcNPR3*	Plants showed resistant to cacoa pathogen *Phytophthora tropicalis*	Fister et al. (2018)
Daucus carota	*F3H*	Leads to discoloration of calli by blocked the anthocyanin biosynthesis pathway	Klimek-Chodacka et al. (2018)
Cucumic sativus	*4E* eIF4E	Increased resistance against the viruses like Cucumber vein yellowing virus, Zucchini yellow mosaic virus, and Papaya ringspot mosaic virus type-W	Chandrasekaran et al. (2016)
Saliva miltiorrhiza	*SmRAS*	Reduced the levels of phenolic acids Rosmarinic acid and lithospermicacid B	Zhou et al. (2018)

applications and successful gene editing experiments. The success of CRISPR-mediated gene knockout or knock-in mainly depends on the PAM) location. The ability of sgRNA to produce null alleles is quite high due to its high efficiency. Synthetic sgRNA binds to the target sequence and leads to cleavage by Cas9 at 3–4 bases after the PAM sequence. Length of sgRNA is about 100 nucleotides (nt) and the 5' end of this 100 nt sequence contains 20 nt gRNA which is critical for targeting the gene of interest with help of PAM sequence i.e. NGG. PAM sequence helps to identify the target site. Normally, target sites that code for amino acids near the N' terminus of the protein are avoided, to mitigate the ability of the cell to use an alternative ATG downstream of the annotated start codon (Chuai et al. 2018; Doench et al. 2016; Wang et al. 2019c). After *in silico* designing, best sgRNA sequences are chosen as per the computational tool-specific prescribed matrix. Selected sgRNAs are synthesized by the oligo synthesis methods.

Multiplexing of the Gene Targets:

The cellular processes in plants are regulated by complex genetic networks. Therefore, the manipulation of traits depends on the precise genetic engineering of multifaceted metabolic pathway, which is required for the desired expression of multiple genes. CRISPR/Cas9-mediated editing offers great potential in multiplexing target editing. Generally, editing of multiple target gene is achieved by two approaches i.e., each gRNA expressed by an individual promoter or multiple gRNAs are expressed under one promoter as a single transcript which is further processed for the editing of the specific target site (Minkenberg et al. 2017). Several studies have been reported for multiple site-directed mutagenesis by using gRNA expression system. These systems incorporate assembly of multiple and individual gRNA expression cassettes in a CRISPR plasmid (Lowder et al. 2015; Ma et al. 2015; Xing et al. 2014). The expression of Cas9 was successfully demonstrated via CRISPR/Cas9-mediated genome editing to improve mutagenesis efficiency in the Arabidopsis genome by using tissue specific-promoter such as egg cells, germ cells or meristematic cells (Gao et al. 2015; Gao et al. 2017; Hashimoto et al. 2018; Hyun et al. 2015; Mao et al. 2016; Nishitani et al. 2016; Osakabe et al. 2016, 2017; Wang et al. 2015b; Yan et al. 2015). Previously, the construction of complex multigene assembly was considered a bottleneck in biotechnology. However, with the development of molecular tools, several new methods that allow the simultaneous assembly of multiple target sites with the marginal scars have appeared from the nascent field of synthetic biology (Casini et al. 2015; Patron 2014). Currently, Type IIS restriction endonuclease- mediated assembly is most widely adopted known as Golden Gate Cloning (Engler et al. 2008, 2014; Weber et al. 2011), and also a ligation-independent methods developed known as Gibson assembly (requires the production of linear overlapping fragments) (Gibson 2011; Gibson et al. 2008a, b).

Vector preparation: Several groups have developed multiple sgRNAs into single Cas9/sgRNA expression vectors by using the Golden Gate cloning or the Gibson Assembly method, in which multiple sgRNAs are driven by separate promoters (Lowder et al. 2017). Successively, Xing et al. (2014) developed a toolkit

(CRISPR/Cas9 binary vector set) for the targeted mutagenesis in plants by using plasmid backbones (pCAMBIA or pGreen), that facilitate stable or transient expression of the CRISPR/Cas9 system in a variety of plants species. This toolkit was validated in *Arabidopsis thaliana* and *Zea mays* plants. Subsequently, (Ma et al. 2015) and (Lowder et al. 2015) exemplified a toolkit that is suitable for the species of interest, in which they created a plasmid backbone having a selectable marker and Cas9 expression system. Ma et al. (2015) successfully developed an assembly of eight sgRNA expression cassettes by using this toolkit and demonstrated in rice plants by targeting multiple sites (46 target loci). These toolkits are shown to be easily applicable for multiplexed targeted mutagenesis, but also require the engineering of a customized backbone for each species. A modular cloning system has developed plasmid toolkit (Type IIS enzyme assembly) known as Golden Gate Modular cloning (MoClo) toolkit (Weber et al. 2011) and GoldenBraid (Sarrion-Perdigones et al. 2013). By using these cloning methods, flexible toolkits for Cas9-mediated targeted mutagenesis has been reported in several plant species such *Nicotiana benthamiana* (Nekrasov et al. 2013; Vazquez-Vilar et al. 2016), *Solanum lycopersicum* (Brooks et al. 2014), *Brassica oleracea,* and *Hordeum vulgare* (Lawrenson et al. 2015). These toolkits allow generating complex cassettes and high number of sgRNA expression systems. After construction of CRISPR libraries, it has to be transformed into the plants by various transformation methods such as *Agrobacterium*-mediated, protoplast, callus bombardment, floral dip, biolistic and microinjection to evaluate the phenotypes (Ramkumar et al. 2020).

15.4 Screening Methods

The CRISPR/Cas9-mediated genome editing provides an efficient, precise, and cost-effective way to interrogate the genome and determining gene function and the relationship between phenotypes and genotypes. Several screening methods have been developed by researchers (Fig. 15.2). One of the high-throughput approaches is pooled sgRNA libraries of CRISPR/Cas9, widely used in high-throughput screening for functional genomics (Kweon et al. 2018). Here we describe various CRISPR library screening methods.

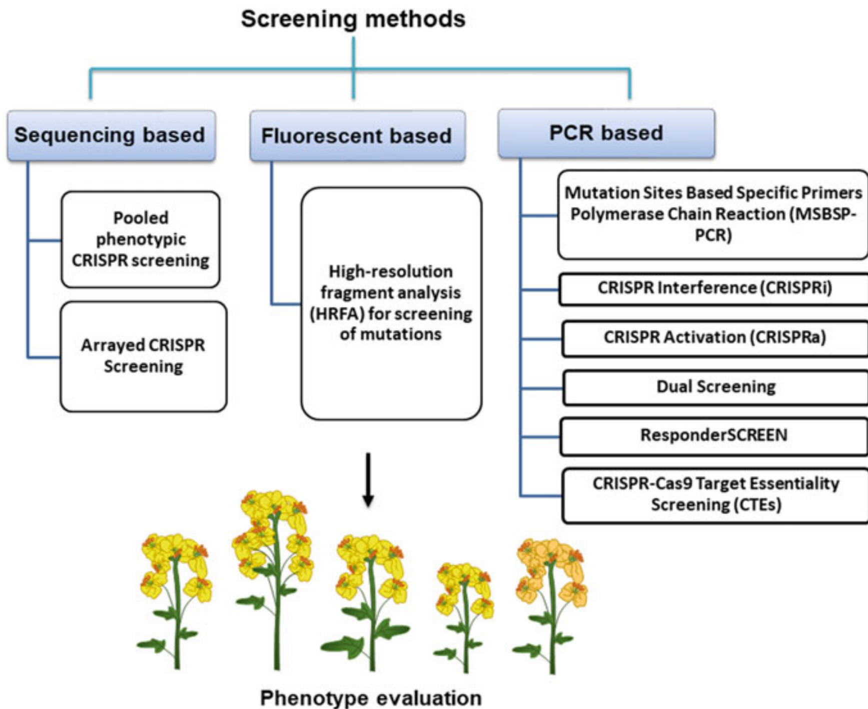

Fig. 15.2 Broad classification of various CRISPR screening methods for evaluation of gene function

15.4.1 Pooled CRISPR Screening

Pooled screening library has been successfully demonstrated in several plants such as rice, tomato, *Arabidopsis*, maize, and soybean. Mutant plants were generated by using CRISPR library and analyzed by visualizing the phenotype and next-generation sequencing (NGS) (Fig. 15.3) (Jacobs et al. 2017; Shalem et al. 2015). Wang et al. (2019b) successfully developed an imaging-based pooled library screening method in the mammalian system to investigate a spectrum of phenotypes, including cell–cell interaction, molecular organization, morphological features, organization of cells, and RNA localization in the nucleus. This approach can be extended to plant protoplasts or unicellular algae to study some of the fundamental aspects of plant biology. For producing the highly efficient library, we have to efficiently target gene of interest in the genome and design appropriate sgRNAs to avoid the off-targets (Costa et al. 2017).

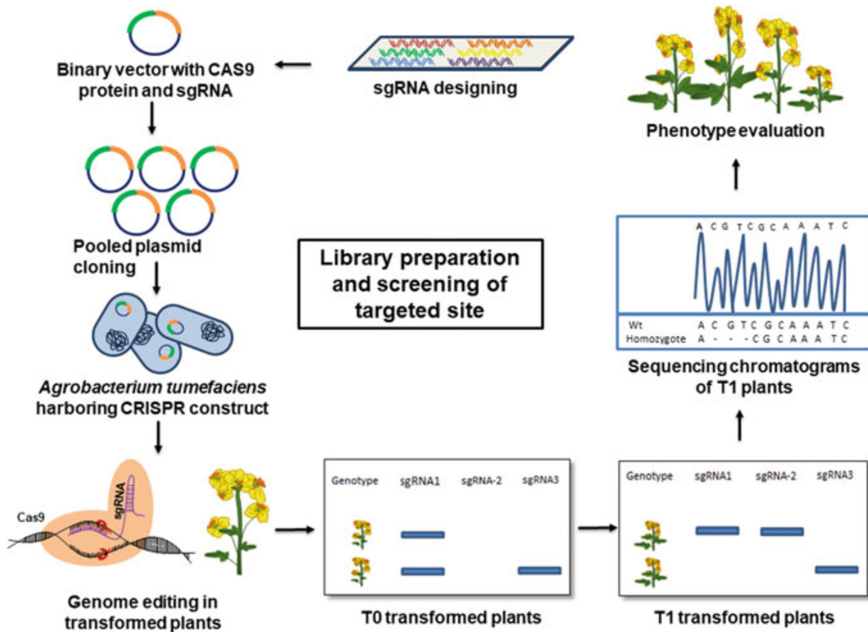

Fig. 15.3 Diagrammatic illustration of *Agrobacterium*-mediated CRISPR pooled library transformation for high-throughput screening. Generation of transgenic plants using CRISPR library and functional profiling of targeted genes through sequencing and phenotypic evaluation is shown

15.4.2 Arrayed CRISPR Screening

Arrayed CRISPR-mediated screening has emerged as an effective alternative to pooled screening, making it possible to explore a wide range of cellular phenotypes that are typically not amenable to pooled screens. To date, arrayed screening has been used to study the broad range of cellular phenotypes such as protein or mRNA localization, stages of cell cycle and cellular morphology by using luminescence, fluorescence or imaging-based assays (Groot et al. 2018; Henser-Brownhill et al. 2017). This method was successfully demonstrated in virus system using viral-based transduction methods, where on a multi-well plate, each well was transduced with a virus that can integrate into the cells (Datlinger et al. 2017; Groot et al. 2018; Hultquist et al. 2016; Kim et al. 2018; Strezoska et al. 2017). Recently, Sercin et al. (2019) demonstrated a high-throughput solid-phase transfection platform for arrayed CRISPR screen human primary cells, to study oncogene addiction in tumor cells.

The arrayed CRISPR screen (solid-phase transfection platform) accomplished two major steps—firstly preparation and storage of transfection mixes and, secondly cell seeding and assays on pre-coated plates. In the first step, establishment of solid-phase transfection with gRNA was done in which the microplate coated with transfection reagents (lipid reagent, sucrose, and gelatin) and synthetic gRNAs were used. After

coating with the transfection mix, plates for freeze-dried, and these complexes can be stored for a longer period or used directly for the cell seedling. In the second step, cell seedling and assays with a wide range of assays such as flow cytometry, microscopy, and cell viability are conducted (Sercin et al. 2019).

15.4.3 High-Resolution Fragment Analysis (HRFA) for Screening of Mutations

Fragment analysis is capillary electrophoresis (CE) based methods. It is an independent enzymatic cleavage and very simple two-step protocols (PCR amplification followed by capillary electrophoresis). Simon et al. (1998) demonstrated high-resolution fragment analysis to detect T-cell receptor γ-chain gene rearrangement in lymphoproliferative disease. Recently, multiple CE-based assays have been developed, which are fast, sensitive, precise, and cost-effective approaches to analyze the CRISPR/Cas-mediated mutation in plants (Lonowski et al. 2017). Andersson et al. (2017) analyzed a large number of genome-edited potato (*Solanum tuberosum*) plants by using high-resolution fragment analysis, in which they detected indels as small as 1 bp and multiple mutated alleles with indels of different sizes.

15.4.4 Mutation Sites-Based Specific Primers Polymerase Chain Reaction (MSBSP-PCR)

MSBSP-PCR is a simple and cost-effective method for the screening of CRISPR/Cas-induced mutation in homozygous/biallelic plants (Guo et al. 2018). This method successfully identified mutants generated by CRISPR/Cas system and efficiently implemented in *Arabidopsis* and tobacco plants. The principle of the MSBSP-PCR method is to identify CRISPR/Cas9-induced mutants in plants. To accomplish this, total genomic DNA was isolated from transgenic plants (T_0 or T_1) and purified, and subjected to first PCR amplification using locus primer set (forward primer and reverse primers). In secondary PCR, amplified product was used as a template to amplify the target site using target-specific (forward primer) and locus-specific (reverse primer) (Guo et al. 2018). Several methods are already available to analyse CRISPR/Cas-mediated mutation, but all methods have advantages and disadvantages. The major advantage of these methods are low cost, technical simplicity, being faster, and accurate and reproducible PCR results, due to two-rounds of PCR. But, with the high complexity genomes such as polyploid species (*Brassica*, cotton, wheat, etc.), during PCR amplification, direct use of primer set, and genomic DNA as a template may cause much non-specific amplification (Guo et al. 2018).

15.4.5 CRISPR Interference (CRISRi) Screening

CRISPR interference (CRISPRi) is a powerful RNA-based technique complementary to CRISPR/Cas9, for target-based silencing of transcription in bacteria, mammals, and plants. Complementing to CRISPR/Cas9, CRISPRi also requires Cas9 but in its catalytically inactive version i.e., dCas9. The new version of catalytically inactive Cas9 is generated *in vitro* by making point mutation at RuvC-like (D10A) and HNH nuclease (H840A) domain (Larson et al. 2013). Similarly, (sgRNA) a 20 bp customized complementary region to the gene of interest can efficiently silence the gene when assembled with dCas9. The sgRNA derived from *S. pyogenes* is 120 nt long chimeric non-coding RNA, consisting a target-specific complementary region of 20 nt, a Cas9 binding RNA structure of 42 nt and lastly a transcriptional terminator of 40 nt. Based upon the engineered sgRNA, CRISPRi can display transcriptional blockage at its elongation site or initiation site (Larson et al. 2013). When sgRNA-dCas9 ribonucleoprotein complex binds to the non-template region of DNA strand of the protein-coding region or UTR, it blocks the elongation process, leading to constraining the transcription. Likewise, the complex binds to the RNA binding site or promoter region i.e. −35 or −10 boxes or *cis*-acting transcription factor binding site (TFBS) or even the trans-acting TFBS, transcription process comes to halt by not allowing the transcriptional machinery bind to the locus (Burden and Weng 2005). The blocking of transcription can be reversed by fusing the transcription factor or RNA polymerase subunit with nuclease null dCas9 or dCas9-sgRNA complex, to direct the target gene promoters (Rousset et al. 2018). Single or multiple mismatches in the sgRNA can bring efficient halt in the process or just using multiple sgRNA can do the same. In CRISPRi, the multiplexed system is more powerful than the single site, as it allows multiple 'knockdown' and 'knockout' in *Saccharomyces cerevisiae* strain (Lv et al. 2015; Stovicek et al. 2015; Zalatan et al. 2015). Thus, this system has the potential and acts as a platform to repress gene expression owing to specificity and efficiency, without altering the DNA sequence of the genome. Along with this, CRISPRi performance can be predicted and have ease in designing, making it cheaper and faster, unlike other previously used systems. CRISPRi has been successfully used in transcriptional repression of the target gene in *E. coli* (Qi et al. 2013) and *S. cerevisiae* (Gilbert et al. 2013) and also demonstrated in tobacco plant (Mercx et al. 2016). However, there are few limitations in the system, firstly requirement of NGG PAM motifs for *S.pyogenes* dCas9 limits its efficiency to the target. However, homologues Cas9 usage has increased the scope to access various PAM sequence (Cong et al. 2013; Sapranauskas et al. 2011). *S. pyogenes* dCas9 can also recognize NAG PAM sequences, which might increase both target sites in the genome as well off-target sites. Therefore, taking benefit from homologues Cas9 with different PAM sequences or using different PAM sequences can significantly reduce the off-target effect and increase targetable space, making it more flexible (Tycko et al. 2016). Secondly, due to local DNA conformation, regulation of DNA in eukaryotic systems such as plants and mammals, an in-depth understanding should

be made, which will increase the repression efficiency (Westra et al. 2012). Lastly, due to short length sgRNA sequence, proportion of off-target binding increases when a long length genome is considered. Thus, mismatches, length and GC contents of gRNA decreases off-target effect (Hajiahmadi et al. 2019).

15.4.6 CRISPR Activation (CRISPRa) Screening

CRISPR transcription activation (CRISPRa), unlike CRISPRi is gain of function gene technology. In which, transcriptional activators are recruited via sgRNAs and nuclease null dCas9 to transcriptional start sites (TSSs) of endogenous genes to induce their overexpression (Zheng et al. 2018). Sometimes more than one transcriptional activator is recruited to TSSs with a single sgRNA to achieve high levels of overexpression (Kampmann 2018). The key to CRISPRa success is designing the nuclease null dCas9 which can explicitly increase the transcriptional rate. This can be done by directly fusing the activator to the dCas9 (Kampmann 2018). There are many strategies to accomplish CRISPRa, for instance by VPR approach (tripartite fusion of VP64 and the activation domains of the p65 subunit of NFκB and Epstein−Barr virus R transactivator, Rta), which involves fusion to -C or -N terminal, showing three to fivefold increase in the expression of the target gene. Other approaches involve: fusion with the tetraloop or stem-loop 2, which excessively upregulates transcription by 12-fold or recruiting the activator using sunTag to create a potent transcriptional activator by recruiting multiple copies of domains to CRISPR/Cas9 system, or using a single variable fragment targeting GCN4, which can tandemly recruit multiple copies of activators or synergistically activator mediator (SAM) approach (Chavez et al. 2015; Jensen 2018; Konermann et al. 2015). In intergenomic variability, basal transcription factor or epigenetic modification largely determines the upregulation of the gene. Intergenomic variability can be achieved by targeting −200 and +1 bp window by SAM (Konermann et al. 2015). Many of the rules for establishing the sgRNA is similar to the CRISPRi but the key difference lies between the two is the window length which is −400 bp to −50 bp upstream to each TSSs. Like CRISPRi, CRISPRa is also specific and nontoxic to the system (Konermann et al. 2015). CRISPRa screening provides a new way of exploring complex genomes to discover diverse transcripts across it. Along with this, it is also likely to provide insights into cellular pathways where redundancy hampers loss-of-function genetic approaches. CRISPRa will also enable the exploration of cellular states in which otherwise inactive pathways are induced, thereby revealing functional coupling within complex cellular networks and suggesting at a potential strategy for fine-tuning gene function in plants.

15.4.7 Dual Screening

Single perturbation from every CRISPR toolbox has been successfully proven to be efficient but does not provide a deeper understanding of each gene in a complex signaling pathway. The dual system activates and represses gene expression by CRISPRa and CRISPRi, two genes simultaneously but asymmetrically targeted by two orthologues Cas9 endonuclease (Boettcher et al. 2018). It has been demonstrated successfully in human chronic myeloid leukemia (CML), where the systems used were constructed with a SunTag-CRISPRa (*Streptococcus pyrogen*) and SaCas9 (*Streptococcus aureus)*, an orthologue of Cas9. These two Cas9 systems have different PAM motif requirements, which makes them non-cross reactive. This system has represented an advance form of genetic construct. In plants system, application of orthologues Cas9 has been demonstrated but not the dual screening like the CML system (Steinert et al. 2015).

15.4.8 ResponderSCREEN

Responder screening is a therapeutic screening method. There are individuals who fail to reciprocate to certain drugs, due to mutation in a specific gene which can alter the sensitivity or resistance to a particular drug. For successful detection of this phenomenon, knocking out an individual gene from the pool may help to identify the target gene. The sgRNA library for the responder screening is collected by CRISPR KO, which follows this comprehensive study (Szlachta et al. 2018). This system of screening owns some advantage that can be stratified to the patient; can maximize the success of the individual drug development program and improve the response outcome (horizon discovery) (Day and Siu 2016). However, this screening-strategy can be used in plants for the fundamental understanding of small molecule- receptors interactions in plants.

15.4.9 CRISPR/Cas9 Target Essentiality Screening (CTEs)

CRISPR-Cas9 target essentiality screening method is related to drug discoveries. Identification of accurate target for the drug makes it a powerful tool in drug discovery. As in RNAi-based (RNA interference), target validation assays can only modulate the gene expression but unable to create a knockout. Using the horizon's CRISPR/Cas9-based medium-throughput target assay can find the essentiality of the drug to the biological target (Grassian et al. 2015; Neggers et al. 2018). The mechanism of CTEs involves transfecting cells with constructs harboring CRISPR/Cas9 system, creating DSBs and then culturing the colonies with the single cell. This follows analysis of the gene by restriction digestion assay, and a detailed in-del analysis in

selected colonies to identify the essential and non-essential targets (Lee et al. 2018). This system shows advantages over RNAi technique.

15.5 Generation of Transgenic Plants Using CRISPR Library

The efficiency of CRISPR-mediated editing in plants facilitates the development of high-throughput mutagenesis. Transformation of plants using pooled CRISPR libraries and creation of transgenic mutant lines can be produced with minimum transformation attempts and in relatively short periods. In recent years, several transgenic plants such as rice (*Oryza sativa*), tomato (*Solanum lycopersicum*), and soybean (*Glycine max*) were generated and evaluated for their phenotype by targeting multiple sites in a genome using CRISPR library (Table 15.3). High-throughput genome-wide mutant libraries have been introduced into rice plants by Lu et al. (2017) and Meng et al. (2017a). Lu et al. (2017) developed a pooled approach for genome-wide mutagenesis of genes in rice using the CRISPR library (sgRNA pooled library). In this approach, they designed a sgRNA library with 88,542 members, targeting 34,234 genes. 84,384 transgenic plants were generated through *Agrobacterium*-mediated transformation of a library of pooled sgRNAs. The transgenic plants were validated through sequencing and the mutation frequency was 83.9% (Lu et al. 2017). Whereas, Meng et al. (2017a) generated 14,000 transgenic rice plants by transforming with *Agrobacterium* having pooled 25,604 sgRNAs, corresponding to 12,802 genes. In the same year, Jacob et al. (2017) reported high efficiency mutagenesis in tomato plants through CRISPR system, using the pooled CRISPR library. In this study, total 54 genes related to the immunity-associated leucine-rich repeat (LRR-RLK) subfamily XII, were targeted by a single transformation using *Agrobacterium* with pooled CRISPR library. 31 transgenic lines were generated, analyzed through PCR and sequenced. Mutations were detected in 15 transgenic plants and the mutation frequency was 62.5%. Recently, Bai et al. (2019) demonstrated *Agrobacterium*-mediated pooled CRISPR library transformation platform in soybean plants, which created 407 transgenic lines, which were analyzed by PCR and sequencing. The analyzed transgenic lines showed an average mutagenesis frequency of 59.2%.

Table 15.3 Genome-wide pooled sgRNA CRISPR library screening in plants

Crops	Screening method	Total no. of targeted genes and sgRNA	Reference
Oryza sativa	sgRNA pooled	34,234, 88,541	Lu et al. (2017)
Oryza sativa	sgRNA pooled	12,802, 25,604	Meng et al. (2017a)
Solanum lycopersicum	sgRNA pooled	18, 54	Jacobs et al. (2017)
Glycine max	sgRNA pooled	102, 70	Bai et al. (2019)

Currently, the complete genomes of many plants have been sequenced. So, these high-throughput genome-wide pooled CRISPR library transformation strategies can be useful for other plants as well. CRISPR library can also be made for plants without the availability of genome sequence, using semi-random primers for mRNAs (Arakawa 2016).

15.6 DNA-Free Genome Editing

The CRISPR-mediated genome editing system has already shown its efficiency, simplicity and versatility in several applications in plants, microbes, human, and animal systems. For CRISPR-mediated improvement of crops, it is desirable to develop the final product without transgenes to minimize regulatory issues. CRISPR-mediated transgenes may be eliminated through conventional breeding techniques and screening the segregating populations.

Mutated plants without transgene integration can be achieved using CRISPR-Cas9 ribonucleoprotein complexes (RNPs). CRISPR-Cas9 RNPs have been successfully delivered into plants through PEG-mediated (Polyethylene glycol) or particle bombardment transformation in *Arabidopsis*, tobacco, lettuce, rice, apple, grape, petunia, soybean, *Brassica*, wheat, and maize (Kim et al. 2017; Liang et al. 2017; Malnoy et al. 2016; Murovec et al. 2018; Subburaj et al. 2016; Svitashev et al. 2016; Woo et al. 2015). The major advantage of RNP delivery is eliminating the introduction of foreign DNA and curtailing random DNA integration into the genome.

15.7 Conclusion and Future Implications

Various CRISPR-based methods contributed to forming a broad platform for conducting complex screens where genes can be knocked in, knocked out, knocked down, or even activated at the same time in a single cell or organism. CRISPR/Cas gene-editing system has advantages when it is combined with next-generation sequencing, by which researchers can easily do the comprehensive mutational screening. Optimization and proper designing of gRNAs is very important at every stage to avoid deleterious effects of off-target editing. The major advantage of CRISPR-based library screening is higher specificity, multiplexing, and high-throughput gene targeting. To nullify the false positive/negative results, library preparation quality check is required at each point of the screening procedure. Analyzing gene sequence or expression with the help of above described methods is very useful for identifying the function of the gene(s).

Acknowledgements This work was supported by the Department of Biotechnology (Grant No. BT/PR26019/GET/119/107/2017), Government of India to SKL.

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Chapter 16
CRISPR/Cas9 System, an Efficient Approach to Genome Editing of Plants for Crop Improvement

Saber Delpasand Khabbazi, Afsaneh Delpasand Khabbazi, Volkan Cevik, and Ali Ergül

Abstract Crop production in agriculture is affected by plant genetic background and environmental factors. The application of modern molecular biology tools to conventional plant breeding approaches has facilitated the plant genetic improvement attempts. After the extensive employment of recombinant DNA technology in diverse plant species and despite achievements, the use of transgenic crops has been encountered with public concerns due to the presence of transgenes. The advent of sequence-specific nuclease-based editing technologies especially clustered regularly interspaced short palindromic repeat-associated protein system (CRISPR/Cas) has opened a promising avenue in genetic engineering of plants. The importance of this approach is emphasized since it is a simple and robust tool, moreover, non-transgenic mutants can be selected in later generations. Following the successful use of the CRISPR/Cas9 editing tool in model plants, the applications of this system have been increasingly reported in different plant species. This chapter reviews the contribution of the CRISPR/Cas9 system in the development of genetically modified crops with improved yield, nutritional value, and response to biotic and abiotic stress factors.

Keywords Abiotic stress factors · Biotic stress factors · Genome editing · Improved yield · Non-transgenic · Nutritional quality · Transgenic technology

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G. Tang et al. (eds.), *RNA-Based Technologies for Functional Genomics in Plants*,
Concepts and Strategies in Plant Sciences, https://doi.org/10.1007/978-3-030-64994-4_16

16.1 Introduction

Food demand is the basic requirement of life and since the early ages of agriculture, farmers have attempted to enhance the quality and yield of plant species. However, agricultural products are threatened by biotic and abiotic stress factors. Global climate change and the emergence of new genotypes of plant pests and pathogens pose serious threats to the sustainable production of crops. Besides, the growing world population which is estimated to reach 9.7 billion by 2050 and the increase of 58–98% food demand during this time renders the conventional agricultural practices insufficient to secure the food supply (Valin et al. 2014). Therefore, the introduction of innovative technologies not only contributes to crop production by improving the tolerance of crop plants during stresses, but also improves the nutritional quality and yield of crops. The advent of recombinant DNA technology tackled many existing restrictions in plant breeding stages. Identification, characterization, and transformation of foreign genes into host plants, to confer desired features including resistance to biotic and abiotic stress factors, herbicide tolerance, improvement of nutritional qualities and yield, have been numerously reported (Pasquali et al. 2008; Tripathi 2012; Bakhsh et al. 2015, 2016; Khabbazi et al. 2016, 2018; Anayol et al. 2016). Modification of the plant genome using chemical and physical mutagenesis is another approach to achieve desired agricultural traits (Roychowdhury and Tah 2013). However, plant genetic modification through these mutagens has random effects on the host plant and consequently might lead to an unexpected outcome. To reduce the unwanted genome alterations, target-specific modifying tools are advantageous and could be exploited alternatively. Genome editing of plants has gained notable achievements since the advent of sequence-specific nucleases (SSN) (Shelake et al. 2019). These tools including zinc finger nucleases (ZNFs), transcription activator-like effector nucleases (TALENs), and clustered regularly interspaced short palindromic repeat (CRISPR)/Cas9 system, have encouraged the creation of plant cultivars with desired traits. ZNFs- and TALENs-based genome editing has low-efficiency, is technically complex and cumbersome, therefore despite the reported researches (Lor et al. 2014; Sawai et al. 2014; Clasen et al. 2016) and due to the existing limitations, these methods are not employed any further (Kumar et al. 2018). CRISPR/Cas9 technology on the other hand is more accurate, simple and simultaneously can modify several targets in the genome. Moreover, improvement of a trait in crops using genome editing-based breeding methods requires an average of 4–6 years, while crossbreeding, mutation breeding, and transgene breeding approaches require 8–12 years (Chen et al. 2019). The discovery of CRISPR/Cas9 and its application in the determination of gene function and plant genetic modification studies have opened a promising avenue for the increased food supply in current global conditions (Yan et al. 2016; Minkenberg et al. 2017). The importance of this system is highlighted especially when it induces heritable targeted mutagenesis and contributes to the development of transgene-free plants (Wang et al. 2014; Pan et al. 2016). The introduction of non-transgenic edited

plants has an unprecedented advantage to develop plants with desired traits, however maintenance of these genotypes is crucial as well. Hence, in vitro multiplication and generation of synthetic seeds could ensure the conservation and availability of the valuable genotypes (Ergül et al. 2018; Khabbazi et al. 2017, 2019). This chapter reviews the major CRISPR/Cas9-based genetic modifications in different plant species with improved yield, nutritional quality, and response to biotic and abiotic stress factors.

16.2 CRISPR/Cas System; Origin, Mechanism, and Its Use in Genome Editing

CRISPR/Cas is an adaptive immunity system naturally existing in bacteria and archaea and protects the host from invasive genetic elements like phages. Small nucleic acid fragments of invasive pathogens are incorporated into the host's CRISPR loci (called spacer) and stored for future encounters (Amitai and Sorek 2016). Once the host cell is exposed to a new invasion, spacer sequences are transcribed, and as individual CRISPR RNAs (crRNAs) guide the Cas nuclease to the cognate nucleic acid sequences of the pathogen and cleave them (Barrangou et al. 2007). Based on the nature of interference and molecules involved, CRISPR/Cas systems have been divided into two classes and six types. CRISPR/Cas systems can target DNA (type I, II, V), RNA (type VI) or both DNA and RNA molecules (type III) (Koonin et al. 2017). Type II CRISPR/Cas9 isolated from *Streptococcus pyogenes* was the first demonstrated to show target-specific cleavage on the genome in eukaryotic cells and under in vitro conditions (Gasiunas et al. 2012; Jinek et al. 2012; Mali et al. 2013; Cong et al. 2013). This system is based on RNA-guided interference with DNA and has been employed in the development of plants with desired traits. CRISPR/Cas9 complex is comprised of Cas9 nuclease and a single guide RNA molecule (sgRNA). The sgRNA is an artificial fusion of a crRNA with a fixed transactivating crRNA (tracrRNA). A 20-nucleotide fragment at 5′ end of the sgRNA directs the complex to the target sites in genome adjoining to conserved protospacer-adjacent motifs (PAMs). The Cas9 enzyme induces double-strand breaks (DSBs) at specific genomic sites and exposes the broken DNA to either error-prone non-homologous end joining (NHEJ) repair or the precise homology-directed repair (HDR) mechanism. NHEJ may cause some unwanted indel mutations at the junction site of the repair whereas the HDR pathway stimulates accurate alteration of a gene sequence. HDR-mediated insertion or replacement of desired sequences in target sites of the genome provides an unprecedented tool for the genetic engineering of plants (Voytas and Gao 2014). Although, delivery of template DNA together with preparation of DSBs makes HDR mechanism more challenging than NHEJ, in vitro and *in planta* studies have reported the HDR-mediated genome editing in plants (Zhang et al. 2018a).

16.3 Effectiveness and Versatility of CRISPR/Cas9 System in Genome Editing of Plants

The use of CRISPR/Cas9 in genome editing of plants was first indicated in model plants tobacco (*Nicotiana benthamiana*) and Arabidopsis (Li et al. 2013; Nekrasov et al. 2013). The versatility of CRISPR/Cas9 based genome editing has been studied by knocking out the phytoene desaturase gene (*PDS*) in different crop species such as potato, grape, sweet orange, and watermelon. Disruption of the *PDS* gene causes albino phenotype in mutants and acts as a visual marker for CRISPR/Cas9-based mutagenesis (Jia et al. 2014; Pan et al. 2016; Tian et al. 2017; Nakajima et al. 2017). The inheritance pattern of the *pds* mutants was investigated by monitoring the albino phenotype, sequencing, and genotyping (Pan et al. 2016). The proportion of *PDS* mutated cells is correlated with the cell age so that lower leaves of the plant display higher rates of the *PDS* mutation (Nakajima et al. 2017). The higher rates of mutagenesis in older leaves could be due to inefficient repair mechanism of old cells or the repeated induction of double-strand breaks (DSBs). The induced *PDS* mutagenesis in watermelon plant caused the albino phenotype either as a clear or mosaic pattern with no off-target effects (Tian et al. 2017). Transient expression of CRISPR/Cas9-gRNA-*PDS* in sweet orange leaves also disrupted the *PDS* gene without detecting any off-target effects. Pretreatment of the sweet orange leaves with a culture of *Xanthomonas citri* ssp. *citri* facilitated the agro-infiltration and enhanced the protein expression level in leaves (Jia and Wang 2014). To increase the efficiency of mutagenesis, studies have been carried out to optimize the Cas9 gene expression. Comparing zCas9 (Xing et al. 2014), AteCas9 (Schiml et al. 2014; Fauser et al. 2014) and Cas9p (Ma et al. 2015), AteCas9 was identified as the most efficient codon-optimized Cas9 enzyme in knocking out the flavanone-3-hydroxylase gene in carrot cells (Klimek-Chodaka et al. 2018). Numerous reports have supported the use of CRISPR/Cas9 for accurate mutagenesis in a variety of crop species with different aims of nutritional quality improvements or biotic and abiotic factor tolerance (reviewed in Ricroch et al. 2017; Santosh Kumar et al. 2020).

16.4 Genome Editing Technologies Contribute to Pathogen Resistance in Crops

Pathogens along with insect pests threaten sustainable crop production worldwide. Bacteria, viruses, and fungi are the most devastating pathogens causing serious economic losses (FAO 2017). Approximately 20–40% of global agricultural losses are caused by these pathogens (Savary et al. 2012). In certain crops such as rice, diseases caused by pathogens are the main reason for the yield losses (Heinrichs and Muniappan 2017). To meet the food demands of the growing global population, chemicals have traditionally been used to combat pathogenic diseases. Different aspects of chemical control from consumer health to imposed costs have brought the

need for alternative approaches. Using classical plant breeding methods, resistant cultivars have been developed, however, it is a time-consuming and tough process. In the last two decades, the transformation of resistance genes has successfully enhanced the resistance of crops to pathogens. The gradual acquisition of pathogen resistance to chemicals and resistant varieties can render these approaches ineffective. Genome editing-based technologies have opened new avenues to control agricultural product losses and superseded the limitations of conventional breeding methods. Employment of engineered SSNs such as ZFNs, TALENs, and CRISPR/Cas9 has facilitated the genome modifications toward biotic stresses. Unlike ZFNs and TALENs which are low efficient and technically complex, CRISPR/Cas9 is highly effective and target-specific; however, the efficiency of the entire process remains species- and genotype-dependent. Moreover, off-target mutations, as well as unexpected damages, could be limiting in plant genetic manipulations. PEG-, gene gun- and *Agrobacterium*-mediated transformation methods and lately a nanodot based transformation method have been used to develop different strategies for stable and transient expression of Cas9/sgRNA constructs in crops (reviewed in Borrelli et al. 2018; Doyle et al. 2019). Employment of gene bombardment method ensures the availability of a sufficient amount of template DNA in host cells, however, the existence of excessive foreign genes and vector sequences might be contaminant for the recipient cell. *A. tumefaciens*-mediated transformations of plants results in the stable transformation of gene constructs and screening of the mutant plants containing foreign gene sequences in subsequent stages (Baltes et al. 2017). In the last few years, CRISPR/Cas9 has been widely used to improve the resistance of crops to different biotic stresses. These studies have mainly addressed viral, fungal, and bacterial diseases in plants (Table 16.1).

16.4.1 CRISPR/Cas-Based Engineering of Crops for Virus Resistance

Most of the biotic resistance studies in CRISPR-based edited plants are related to viruses (Borrelli et al. 2018). Based on their genome nature, plant viruses are classified into five groups: single-stranded DNA, single-stranded RNA, double-stranded RNA, positive-sense single-stranded RNA, negative-sense single-stranded RNA, and reverse-transcribing viruses. Virus-resistant plant species could be created through either targeting the viral genetic material or editing plant genome. Resistance obtained by disruption of viral genes requires the integration and permanent expression of Cas9/sgRNA constructs, therefore, such modifications subject the plants generated to biosafety regulations of genetically modified organisms (GMOs). In contrast, modifying plant susceptibility genes such as eukaryotic translation initiation factors necessary for the RNA virus life cycle, release non-transgenic virus-resistant plants (Sanfcon 2015).

Table 16.1 CRISPR/Cas-based editing of plant genome and development of plants with resistance/tolerance to biotic stress factors

Biotic stress factors	Plant species	Target genes	Results obtained	Reference
Virus	*Cucumis sativus*	*eIF4E*	Ipomovirus immunity, tolerance to the ZYMV and PRSMV-W	Chandrasekaran et al. (2016)
	Oryza sativa L. japonica	*eIF4G*	RTSV	Macovei et al. (2018)
	Nicotiana benthamiana	TYLCV coding/non-coding sequences	Resistance to TYLCV	Ali et al. (2015)
	N. benthamiana	*AGO2*	Tolerance to various viruses including PVX, TuMV, and TCV	Ludman et al. (2017)
	Arabidopsis thaliana	*eIF(iso)4E*	Resistance to TuMV	Pyott et al. (2016)
	A. thaliana and N. benthamiana	BSCTV coding/non-coding sequences	Tolerance to BSCTV	Ji et al. (2015)
	N. benthamiana	LIR and Rep/RepA	BSCTV	Baltes et al. (2015)
	N. benthamiana	CP, Rep, and IR	CLCuKoV, MeMV, and TYLCV	Ali et al. (2016)
	N. benthamiana	GFP1, GFP2, HC-Pro, CP	TuMV	Aman et al. (2018)
	A. thaliana and N. benthamiana	ORF1a, 2a, 2b, 3a, ORF-CP, and 3’UTRs	CMV, TMV	Zhang et al. (2018b)
Fungus	*O. sativa*	*OsERF922*	Tolerance to Blast	Wang et al. (2016)
	Solanum lycopersicum	*SlMlo*	Resistance to powdery mildew	Nekrasov et al. (2017)
	Triticum aestivum	TaMLO-A1	Resistance to powdery mildew	Wang et al. (2014)

(continued)

Table 16.1 (continued)

Biotic stress factors	Plant species	Target genes	Results obtained	Reference
	T. aestivum	*EDR1*	Resistance to powdery mildew	Zhang et al. (2017)
	Vitis vinifera	*MLO-7*	Resistance to powdery mildew	Malnoy et al. (2016)
	V. vinifera	*WRKY52*	Resistance to gray mold	Wang et al. (2018)
	Theobroma cacao	NPR3	Resistance to Black pod disease	Fister et al. (2018)
	O. sativa L. *japonica*	SEC3A	Resistance to Rice blast disease	Ma et al. (2018)
Bacteria	*O. sativa*	*OsSWEET13*	Tolerance to bacterial blight	Zhou et al. (2015)
		SWEET gene promoters (SWEET11/SWEET13/SWEET14)	Broad-spectrum resistance to bacterial blight	Oliva et al. (2019)
	Citrus sinensis Osbeck	CsLOB1 promoter	Resistance to Canker	Peng et al. (2017)
	Citrus paradisi	PthA4 effector binding elements in the Type I CsLOB1 promoter	Tolerance to citrus canker	Jia et al. (2016, 2017)
	S. lycopersicum	*SlJAZ2*	Resistance to bacterial speck	Ortigosa et al. (2018)
	Malus domestica	*DIPM-1, DIPM-2,* and *DIPM-4*	Resistance to Fire blight	Malnoy et al. (2016)

CRISPR/Cas edited virus-resistant plants have been mostly developed for resistance to geminiviruses (Ji et al. 2015; Baltes et al. 2015; Ali et al. 2015, 2016). Geminiviruses cause substantial destructions in important families of plants including *Fabaceae*, *Cucurbitaceae*, *Solanaceae*, etc. (Zaidi et al. 2016). Most of these studies have been performed on model plants *Arabidopsis thaliana* and *Nicotiana benthamiana* (Table 16.1). Expression of Cas9/sgRNA constructs in host plants targeting the coding and non-coding regions of the virus reduced the virus accumulation. Targeting the virus coat protein (CP), replication protein (Rep) and intergenic regions (IR) effectively attenuates viral symptoms; however, mutations disrupting the virus in the non-coding IRs such as the stem-loop sequence within the origin of replication resulted in more reduction of virus replication and accumulation

(Ali et al. 2015). Furthermore, targeting the virus in the IR inhibits the creation of new variants of the mutated virus which can potentially replicate and escape the CRISPR/Cas9 machinery (Ali et al. 2016). Targeting plant susceptibility genes such as *eIF4E*, *eIF(iso)4E*, and *eIF4G* has resulted in the development of non-transgenic plants resistant to RNA viruses of *Potyviridae* (Chandrasekaran et al. 2016; Pyott et al. 2016; Macovei et al. 2018).

In most of the studies, CRISPR-based edited plants have been developed using the SpCas9 enzyme isolated from *Streptococcus pyogenes* (reviewed in Ricroch et al. 2017). However, this enzyme only cuts double-stranded DNA molecules, therefore, remains inefficient for RNA viruses. Later studies isolated other CRISPR associated enzymes from *Francisella novicida* (FnCas9) and *Leptotrichia wadei* (LwaCas13a) which could target RNA molecules. FnCas9 and LwaCas13a have been used to develop plants resistant to *cucumber mosaic virus*, *tobacco mosaic virus* and *turnip mosaic virus* (Zhang et al. 2018b; Aman et al. 2018). Endonuclease activity of FnCas9 is not essential for enzymatic interference; therefore, FnCas9 could be utilized as a CRISPR interference tool (CRISPRi) (Zhang et al. 2018b).

16.4.2 CRISPR/Cas-Based Genetic Modification of Plants Against Fungal Disease

Studies over the plant-pathogen interactions have elucidated the molecular mechanisms underlying pathogen infection and plant immune system. The presence of plant genes serving pathogens can lead to the emergence of diseases. Targeting the susceptibility genes corresponding powdery mildew (*MLO-A1*, *MLO-1,* and *MLO-7*), transcription factors involved in stress responses (WRKY52 and ERF922), regulators associated with host immune system (NPR3) and subunits of the exocyst protein complex (SEC3A), has conferred resistance to fungal diseases in a variety of annual and perennial plants (reviewed in Borrelli et al. 2018).

Till date, resistance against powdery mildew (Wang et al. 2014; Malnoy et al. 2016; Nekrasov et al. 2017), gray mold (Wang et al. 2018), black pod disease (Fister et al. 2018), and blast disease of rice (Wang et al. 2016; Ma et al. 2018) has been improved in several crops such as wheat, rice, tomato, grapevine, and cacao tree. Self-pollination and subsequent selection of individuals with on-target mutations but lacking Cas9/sgRNA constructs introduce edited non-transgenic plants (Nekrasov et al. 2017). Generally, CRISPR/Cas constructs are transferred to host plants through vector plasmids however this method is intercepted by the biosafety regulations of transgenic crops. Implementing genome editing of plants without using DNA plasmid attempts to reduce the social distrust related to the transformations of foreign genes. Transient expression of plasmid-free genome editing constructs is critical particularly in perennial fruit crops due to the longer time required for segregation and backcrossing. In this regard, Malnoy et al (2016) delivered the purified CRISPR/Cas9

ribonucleoproteins (RNPs) to the protoplast of apple and grape cultivars. This method could increase the public acceptance of the genetically modified (GM) crops and thereby could be exempted from the existing GMO regulations (Waltz 2012; Jones 2015).

16.4.3 Bacterial Resistance Achieved Through CRISPR/Cas9

Unlike other pests and pathogens, bacterial diseases of plants cannot be controlled by chemicals, and the only way to cope is to prevent the disease by using different approaches such as efficient agricultural practices, cultivating healthy plants, and developing resistant varieties (Janse 2001). CRISPR/Cas9-based targeted mutagenesis on plant susceptibility genes has conferred resistance to bacterial infection in host plants. Canker is one of the serious diseases of citrus cultivars around the world. The disease is caused by *Xanthomonas citri* subsp. *citri* (*Xcc*) and *CsLOB1* is the susceptibility gene in the host induced by the pathogenicity factor PthA4 (Hu et al. 2014). Modifying the PthA4 effector binding elements (EBEs) in the promoter region of *CsLOB1* gene in Duncan grapefruit variety interfered with the binding of PthA4, therefore, decreased the typical symptoms of bacterial canker (Jia et al. 2016). Later CRISPR/Cas9-mediated disruption of EBE_{PthA4} confirmed the presence of the link between *CsLOB1* promoter activity and the susceptibility against *Xcc* in Wanjincheng orange (*Citrus sinensis* Osbeck) (Peng et al. 2017).

Expression of *OsSWEET13,* a member of sucrose transporters family proteins, is required for bacteria-host interactions. *OsSWEET13* activity is induced by *Xanthomonas oryzae* pv. *Oryzae* PthXo2 effector protein and CRISPR/Cas9-mediated knockout of *OsSWEET13* susceptibility gene better explored the role of PthXo2 in the emergence of the bacterial blight of rice (Zhou et al. 2015). Recently, CRISPR-based genome-edited *SWEET* gene promoters (*SWEET11*/*SWEET13*/*SWEET14*) introduced rice lines with broad-spectrum resistance to bacterial blight disease (Oliva et al. 2019).

16.5 Tolerance to Herbicides and Abiotic Stress Factors via CRISPR/Cas9

Weeds are the biotic restraint that causes significant losses in agricultural production and if not controlled timely and effectively reduces the crop yield by up to 50% (Oerke 2006). Transgenic soybean, canola, cotton, and corn were the first glyphosate and glufosinate tolerant crops released to the market in 1996–1997. According to a report, more than 100 million hectares worldwide are cultivated with genetically modified (GM) crops with at least one herbicide tolerance gene (ISAAA 2012). Adoption of herbicide-tolerant (HT) transgenic crops reduces the use of chemicals

and greenhouse gas emissions resulting from agricultural practices; moreover, it increases the product yield, farmer income (Cerdeira and Duke 2006; Martino-Catt and Sachs 2008). However, the expression pattern of the transgene is influenced by genetic elements such as the gene promoter and intron fragments (reviewed by Huang et al. 2015). The repeated cultivation of a single herbicide-tolerant crop causes the evolution of weeds resistant to the frequently used chemicals (James 2014). To address this issue, gene stacking has contributed successfully.

Herbicides bind to vital proteins in the host and affect the normal functioning of the cell, inhibit plant growth and consequently make weeds unable to survive (Schonbrunn et al. 2001). Modifying the structure of the target proteins such that their functions are not affected but at the same time it disables the binding of herbicides, is an efficient method to confer tolerance to herbicides. Precise mutagenesis in phosphoenol pyruvate binding site within 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS) is an example in this regard which inhibits the binding of glyphosate to phosphoenol pyruvate. Employment of CRISPR/Cas9 tool enables the accurate modification of target sites in the plant genome (Table 16.2). An example in this regard is the substitutions in T178I and P182A within phosphoenol pyruvate binding (PEP) binding site in *Linum usitatissimum* (Sauer et al. 2016). Comparing the edited plants with wild type flax indicated at the higher levels of tolerance to glyphosate. Likewise, induction of such mutations in EPSPS gene of rice plant confirmed the efficiency of the CRISPR/Cas9, however, based on results, only those individuals containing heterozygous gene were viable and showed tolerance to glyphosate (Li et al. 2016a). In addition to EPSPS, *acetolactate synthase* (*ALS*) gene has been targeted by CRISPR/Cas system to apply mutagenesis with the aim of increasing tolerance to herbicides. Acetolactate synthase is a conserved protein required for biosynthesis of branched-chain amino acids such as valine, leucine, and isoleucine. This protein is a primary target for some classes of herbicides including pyrimidinyl thiobenzoates, imidazolinones, triazolopyrimidines, sulfonylureas, and sulfonyl-aminocarbonyl triazolinones (Baltes et al. 2017). CRISPR-based *ALS* mutations conferred tolerance to such herbicides in *Solanum tuberosum*, *Oryza sativa*, *Zea mays*, and *Glycine max* species (Svitashev et al. 2015; Li et al. 2015; Butler et al. 2016; Sun et al. 2016; Endo et al. 2016; Butt et al. 2017) (Table 16.2).

In addition to the crop losses imposed by weeds, factors such as unfavorable drought, salinity, and temperature induce metabolic stresses that affect the growth of plants. CRISPR/Cas-mediated precise disruption of target sites in the genome has been employed to confer tolerance to stress factors (Table 16.2). Ethylene plays an important role in regulating plant response to water deficits and high temperatures (Hays et al. 2007; Kawakami et al. 2010, 2013). Reducing plants' ethylene biosynthesis or decreasing the sensitivity of plants to ethylene can improve grain yield under drought conditions (Habben et al. 2014; Shi et al. 2015). *ARGOS8* gene is a negative regulator of ethylene responses. In maize, the expression of *ARGOS8* gene is relatively low and therefore a constitutive overexpression enhanced the expression level of *ARGOS8* and consequently led to the increase in grain yield under drought stress (Shi et al. 2015). Utilization of CRISPR/Cas system via the HDR pathway contributed to precise allelic variation in *ARGOS8* thereby enhancing the grain yield

Table 16.2 CRISPR/Cas-based editing of plant genome and development of plants with resistance/tolerance to herbicides and abiotic stress factors

Trait	Plant species	Target genes	Results obtained	Reference
Herbicide	*Solanum tuberosum*	*ALS*	Reduced susceptibility to ALS-inhibiting herbicides	Butler et al. (2016)
	Linum usitatissimum	*EPSPS*	Tolerance to glyphosate	Sauer et al. (2016)
	O. sativa	*C287*	Resistance to the herbicide imazamox	Shimatani et al. (2017)
	O. sativa	*ALS*	Reduced susceptibility to ALS-inhibiting herbicides	Sun et al. (2016), Endo et al. (2016), Butt et al. (2017)
	O. sativa	*EPSPS*	Tolerance to glyphosate	Li et al. (2016a)
	Zea mays	*ALS*	Reduced susceptibility to ALS-inhibiting herbicides	Svitashev et al. (2015)
	Glycine max	*ALS*	Reduced susceptibility to ALS-inhibiting herbicides	Li et al. (2015)
	Manihot esculenta	*EPSPS*	Tolerance to glyphosate	Hummel et al. (2018)
Abiotic stress factors	*Z. mays*	*ARGOS8*	Enhanced grain yield under drought stress	Shi et al. (2017)
	A. thaliana	mir169a	Improvement of drought tolerance	Zhao et al. (2016)
	O. sativa	*GT-1* element located at P OsRAV2	Tolerance to Salt stress	Duan et al. (2016)

under drought conditions (Shi et al. 2017). HDR-dependent mutation of *mir169a* improved the drought tolerance in model plant *A. thaliana* such that more than 50% of mutant plants exhibited tolerance to drought stress whereas no wild individual survived (Zhao et al. 2016).

CRISPR/Cas9 tool has also been used to characterize the transcriptional response of rice *RAV* genes (*OsRAVs*) to salt stress. In this study, the expression patterns of the five members of *OsRAVs* were examined under salt stress, and it was observed that only *OsRAV2* was stably induced by salt treatment. Further analysis on the *OsRAV2* promoter region elucidated that *pOsRAV2* is induced by salt. The GT-1

element located at *pOsRAV2* is necessary for salt induction of the promoter (Duan et al. 2016).

16.6 Improvement of Crop Yield, Nutritional Quality and Storage Using CRISPR/Cas9

The effectiveness of the CRISPR/Cas9 system in the disruption of negative regulators of undesirable traits in plants has led to the improvement of yield, nutritional value, and shelf-life of crops, such that these researches share the highest proportion among the CRISPR-based studies (Table 16.3 and Fig. 16.1). Improving the yield is a crucial step to ensure food supply for the growing population. Crop yield is assessed considering various factors such as grain number, size, and weight as well as tiller number and panicle size. As a quantitative trait, yield depends upon many regulating factors. Disruption of genes responsible for the negative regulation of the aforementioned yield factors has contributed to the development of crops with improved yield quality (reviewed by Chen et al. 2019). Knocking out the *OsGn1a, OsGS3, TaGW2*, *OsGW5*, *OsGLW2*, *TaGASR7, OsDEP1*, *TaDEP1*, and *OsAAP3* genes has indicated the effectiveness of CRISPR/Cas system in improving the crop yield by the creation of targeted loss-of-function mutations in plants (Li et al. 2016d; Liu et al. 2017; Lu et al. 2018; Zhang et al. 2016, 2018c). Simultaneous knock out of three genes including *GW2*, *GW5*, and *TGW6* increased the grain weight in rice which demonstrated the effectiveness of CRISPR/Cas use in trait pyramiding in plants (Xu et al. 2016).

Crop quality traits such as starch, oil, and secondary metabolite content have also been improved by the CRISPR/Cas-based gene editing. Starch produced from crops like potato has many applications in the food and industrial sectors. Starch has two components including amylose and amylopectin and modification of amylose or amylopectin alters the properties of starch (Zeeman et al. 2010). Granule-bound starch synthase (GBSS) is the enzyme responsible for amylose synthesis in many plant species. In potato plant (*Solanum tuberosum*), the GBSS enzyme is encoded by a single locus (*GBSS1*) with four alleles. High amylopectin potato genotypes (Waxy potato) have been developed by gene silencing technologies and traditional mutational breeding methods (Andersson et al. 2003; Muth et al. 2008). Recently, CRISPR/Cas-based multiallelic indel mutations of potato *GBSS* gene reduced the amylose, and increased the amylopectin content of the starch (Andersson et al. 2017, 2018). In *Zea mays,* knock out of the endogenous waxy gene led to the production of grains with high amylopectin content (Waltz 2016). Consumption of cereals with enriched amylose or resistant starch benefits human health and reduces the risks of non-infectious serious diseases such as diabetes (Regina et al. 2006). Using the

Table 16.3 CRISPR/Cas-based editing of plant genome and development of plants with improved nutritional value, yield, and other improvements

Plant species	Target genes	Results obtained	Reference
Salvia miltiorrhiza	*SmCPS1*	Reduction in tanshinones	Li et al. (2017a)
S. tuberosum	*GBSS1*	Modified starch quality (high amylopectin potato starch)	Andersson et al. (2017, 2018)
Papaver somniferum	*4'OMT2*	Reduction in benzylisoquinoline alkaloids	Alagoz et al. (2016)
O. sativa	*SBEIIb*	High-amylose rice	Sun et al. (2017)
Camelina sativa	*FAD2*	Modification of fatty acid composition of seed oil	Morineau et al. (2016), Jiang et al. (2017)
	CsDGAT1 and *CsPDAT1*	Altered fatty acid composition	Aznar-Moreno and Durrett (2017)
Hordeum vulgare cv. "Golden Promise"	*ENGase* gene	Modification of N-glycans in grains	Kapusi et al. (2017)
S. lycopersicum	*RIN*	Inhibition of tomato fruit ripening	Ito et al. (2015)
Z. mays	*Wx1*	High amylopectin content	Waltz (2016)
T. aestivum	*GW2*	Increased grain weight and protein content	Zhang et al. (2018c)
Z. mays	*LIGULELESS1* (*LG1*)	Reduction of leaf angles	Li et al. (2017b)
Taraxacum kok-saghyz	*TK 1-FFT*	Rubber biosynthesis in hairy roots	Iaffaldano et al. (2016)
S. lycopersicum	*SlIAA9*	Generation of parthenocarpic tomato	Ueta et al. (2017)
Brassica oleracea and *H. vulgare*	*BolC.GA4.a* *HvPM19*	Pod shatter and control of dormancy	Lawrenson et al. (2015)
Dendrobium officinale	*C3H, C4H, 4CL, CCR,* and *IRX*	Lignocellulose biosynthesis	Kui et al. (2017)
O. sativa	*OspPGM, OsAGPL4*	Male sterility	Lee et al. (2016)
	CrRLK1LS	Regulation of pollen tube growth and integrity	Liu et al. (2016)
	GW2, GW5, and *TGW6*	Improved grain weight in rice	Xu et al. (2016)

(continued)

Table 16.3 (continued)

Plant species	Target genes	Results obtained	Reference
	CSA	Development of japonica photo-sensitive genic male sterile rice lines	Li et al. (2016b)
	Gn1a, DEP1, GS3, and *IPA1*	Enhanced grain number, dense erect panicles, larger grain size	Li et al. (2016c)
	FON2 and *OsMADS3*	Increase of floral organs	Yasui et al. (2017)
	OsSWEET11	Decreased weight and seed setting percentage	Ma et al. (2017)
	EPFL9	Reduction in stomatal density on the abaxial leaf surface of the edited rice plants	Yin et al. (2017)
	MEGs and *PEGs*	Reduced grain yield, starch conten and seed fertility	Yuan et al. (2017)
	Hd2, Hd4, and *Hd5*	Early flowering	Li et al. (2017c)
	LAZY1	Tiller-spreading	Miao et al. (2013)

CRISPR/Cas tool, mutagenesis was implemented in the starch branching enzyme gene (*SBEIIb*), leading to an increased proportion of amylose and resistant starch in rice grains (Sun et al. 2017).

Modifications of the fatty acid composition of plant oils have also been carried out by the CRISPR genome editing tool. Targeting the *Fad2* (Morineau et al. 2016; Jiang et al. 2017) and *CsDGAT1* and *CsPDAT1* (Aznar-Moreno and Durrett 2017) genes in *Camelina sativa* altered the fatty acid composition of the seed oil. CRISPR/Cas9 tool was used to interfere with the ripening process of tomato and enhanced the fruit shelf-life by targeting *RIN*, *SLALC,* and *lncRNA1459* genes (Ito et al. 2015; Yu et al. 2017; Li et al. 2018). Precise mutations of *RIN*, *SLALC* and *lncRNA1459* genes of tomato interfered with the ripening process and enhanced the fruit shelf-life (Ito et al. 2015; Yu et al. 2017; Li et al. 2018). Other studies carried out with the aim of improving quality, yield, and breeding-related attempts has been summarized in Table 16.3.

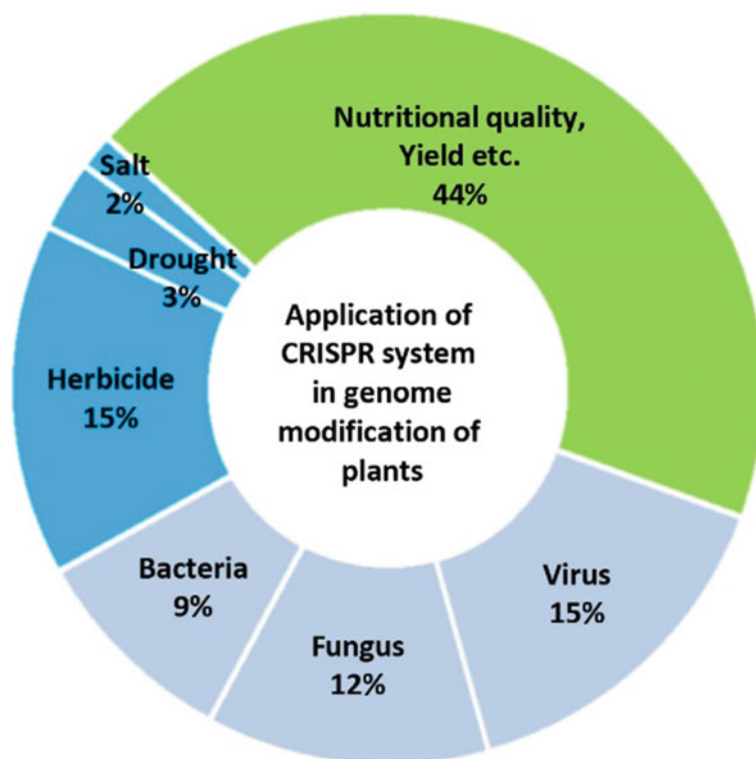

Fig. 16.1 The relative proportion of CRISPR/Cas-based studies in plants

16.7 Conclusion

CRISPR/Cas is a simple, versatile, and robust tool to induce target-specific mutations in different plant species. The employment of the CRISPR/Cas system has indicated an unprecedented contribution to the genome editing of crops. This tool has paved the way for conferring desired characteristics to various plant species and eliminating foreign genes in subsequent generations. Using this technology along with the high throughput system, quantitative traits with polygenic inheritance can be improved by simultaneously targeting the gene loci in crops. CRISPR/Cas system is also useful in the identification of the roles of genetic elements involved in different metabolic pathways. Despite the advances achieved to date, the existence of impediments such as off-target effects, delivery of the CRISPR reagents to host cells, and plant regeneration are still a limiting factor for some species. Unlike the commonly reported knockout-based mutation studies, knock-in researches are less reported and have been carried out on a limited scale. The recent use of the prime editing method in human genome editing has raised the hope for more efficient and precise base editing

attempts without relying upon double-strand breaks or donor DNA (Anzalone et al. 2019). The prime editing technology is also expected to be used for plant cells, which will lead to new directions in the precise engineering of the plant genome (Xu et al. 2020).

Acknowledgments The corresponding author (Dr. Saber D. Khabbazi) is grateful to The Scientific and Technological Research Council of Turkey (TUBITAK-BIDEB) for providing postdoctoral fellowship (Code 2216) to carry out research activities in plant biotechnology at Ankara University.

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Chapter 17
Utilizing RNA-Based Approaches to Understand Plant-Insect Interactions

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Abstract While improving crop plants, plant breeders face several challenges to increase crop yield and protection against abiotic and biotic stresses. Native gene manipulation to generate resistant crop varieties requires efficient tools of modern biotechnology. The elucidations of the function of metabolic pathways and genes have been performed via the reverse genetics approach. However, the instability and incomplete silencing in the reverse genetics approach limit its competence generating aberration in the phenotypic interpretation. Germplasm resistant to imperative biotic stresses is available due to which conventional approach in the breeding of crop plants is performed to control insect-pest and diseases. The wild relatives of crops contain several resistance genes for pest and disease management; however, owing to different poloidy levels, the introgression of these resistant genes in cultivated species through conventional approaches is long and tiresome. Today's advanced biotechnological tools offer several opportunities in the generation of better crops which aid plant breeders to introduce foreign genes to unrelated crop species. In this chapter, we aim to discuss various RNA-based approaches used to comprehend insect-pest management of crops, current development, and future perspectives.

Keywords RNA silencing · MicroRNA · Gene silencing · CRISPR · Genome editing · Plant insect interaction · Biotic stress

17.1 Introduction

RNA silencing is one of the biological processes which causes transcriptional gene silencing (TGS) as well as post-transcriptional gene silencing (PTGS). A highly sequence-specific silencing process, known as RNA interference (RNAi), is conserved among higher eukaryotes. Naturally, RNA interference in plants works as an antiviral defense mechanism, but currently it is being recognized as one of the control strategies in plummeting damaging insect pests of plants (Carthew and

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G. Tang et al. (eds.), *RNA-Based Technologies for Functional Genomics in Plants*, Concepts and Strategies in Plant Sciences, https://doi.org/10.1007/978-3-030-64994-4_17

Sontheimer 2009; Kim et al. 2015; Sen and Blau 2006). RNAi mechanism is triggered by double-stranded RNA (dsRNA) which down-regulates gene expression of the targeted gene and transcripts (Bosher and Labouesse 2000; Kim and Rossi 2007). RNAi technology for crop improvement also poses few biosafety concerns and is recognized as a latent alternative since in the transgenic lines there is a reduced expression of transgene protein (Rajam et al. 2007). In most of the eukaryotic organisms, silencing of a gene is elicited by a small family of RNAs called short-non coding RNAs (snRNAs) (Baulcombe 2004; Vaucheret 2006). Mainly, plants have two classes of small RNAs named as microRNAs (miRNAs) and small-interfering RNAs (siRNAs) having different functions and mode of biogenesis (Carrington and Ambros 2003; Axtell 2013). Correspondingly, both are generated after the RNase III enzyme family Dicer-like (DCL) cleaves the dsRNA precursors (Bernstein et al. 2001; Hutvagner et al. 2001). Lastly, the siRNAs and miRNAs combine RNA induced silencing complex (RISC), argonaute (AGO) protein as well as some protein effector molecules which render downregulation of the desired gene (Mittal et al. 2011). This chapter focuses on utilizing RNA-based approaches to comprehend plant-insect interactions.

17.2 Revolution in RNA Silencing

The phenomenon of co-suppression was discovered in 1990 when the *chalcone synthase* gene was introduced in *Petunia hybrida* plants to enhance the expression of anthocyanin pigments. As a result, transgenic *Petunia* plants produced white flowers rather than the dark purple pigmented flowers. It was concluded that transgene got inserted in the genome but was inactive in its function, which also inhibited the expression of the endogenous gene (Napoli et al. 1990; Hannon 2002; Campbell and Choy 2005). Likewise, another phenomenon similar to co-suppression in *Neurospora crassa* (a fungus), termed quelling, was revealed (Romano and Macino 1992; Cogoni et al. 1996). Afterward, for the first time in 1998, the term RNAi came into illustration when it was revealed in *Caenorhabditis elegans* after injecting dsRNA nematode (twitched) showing different phenotypic appearances (Fire et al. 1998).

17.3 siRNA and miRNA

siRNA and miRNA are recognized as key regulators of gene expression (Bartel 2004). However, both siRNAs and miRNAs show resemblance in size around 20–24 nt but both snRNAs have different structures, biogenesis pathways and modes of actions (Axtell 2013). Moreover, miRNAs and siRNAs are both generated from long RNA precursors through the DCL enzyme (Lee et al. 1993; Bernstein et al. 2001).

17.4 siRNA

RNAi mechanism in plants is initiated by siRNAs; these small interference RNAs are 21–24 nt size generated by cleavage of long dsRNAs, which can be endogenous and exogenous in origin, through Dicer enzyme (Zamore et al. 2000; Hamilton and Baulcombe 1999). siRNAs are then integrated into RISC along with AGO and other effector proteins (Baumberger and Baulcombe 2005; Vaucheret 2008). The multi-protein RISC unwinds double-stranded small-interfering RNA complex. Sense (passenger) and antisense (guard) strand of siRNA duplex are separated, the passenger (sense) strand gets degraded through RNA-helicase activity, while guard strand of siRNA gets engaged with RISC complex (Kusaba 2004). RISC along with antisense (guard) strand acts as a template to target the homologous-mRNA through complementary base pairing and ultimately destroys the target mRNA. The outcome is little or no expression of target gene (Bartel 2004) (Fig. 17.1).

17.5 miRNAs

miRNAs are produced by RNaseIII enzyme, typically DCL1, from single-stranded RNA (ssRNA) with a peculiar stem-loop structures (hairpins). DCL1 processes ssRNA consisting of a stem-loop structure in two steps, to form miRNA and miRNA* duplex. After processing in the nucleus, miRNA duplexes is transferred to cytoplasm and incorporated into multi-protein complex RISC where AGO1 is the central component. Primarily in the miRNA biogenesis pathway, AGO1 and DCL1 are involved (Axtell 2013; Vaucheret et al. 2004). Gene expression is regulated by miRNAs through cleavage of a sequence-specific desired transcript or translational suppression of targeted transcript (Llave et al. 2002; Brodersen et al. 2008). Therefore, miRNAs have an important role in gene expression regulation during plant development (Palatnik et al. 2003; Chen 2012). The site of miRNA biogenesis differs in the case of plants and animals. DCL1 is accountable for both of the processing steps occurring in the nucleus in the case of plants; whereas in animals, Drosha acts in the nucleus during the first step in the process, followed by cleavage from the Dicer in the cytoplasm (Moran et al. 2007). However, miRNAs also have an important role in plants' adaptation to various abiotic stresses (Chiou et al. 2006; Sunkar et al. 2007). The vital role of miRNAs in plant immunity was first identified in *Arabidopsis* plants treated with flagellin derived-elicitor (flg22) elicitor. The function of flg22 was to induce miR393 expression which regulates the F-box auxin receptor negatively, also known as transport inhibitor response (TIR). miR393 suppressed auxin signaling enabling the plant to develop resistance against bacteria (Navarro et al. 2006) (Fig. 17.1).

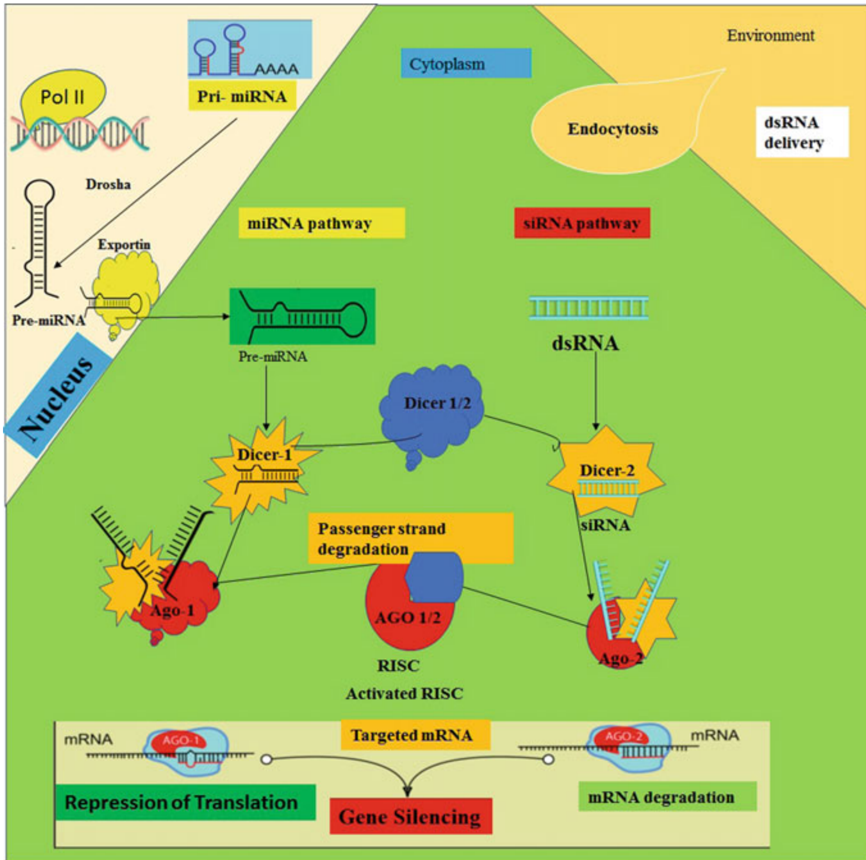

Fig. 17.1 Mechanistic overview of RNAi Pathways (siRNA and microRNA) (Modified from Zotti and Smagghe 2015)

17.6 Factors Affecting the Efficiency of RNAi

As reported in the literature, there are five most important factors that influence the efficiency of the RNAi approach; its silencing is used in achieving control of insect-pest that damages the plants. These factors include:

17.7 Targeted Nucleotide Sequence

According to Araujo et al. (2006), the desired sequence used for knockdown in the targeted organism can silence other off-targets, i.e., insects which are important for the environment. Therefore, in triatomic bug (*R. prolixus*), off-target knockdown

was stated along with the target gene named ‘*nitroporin 2*’. Downregulation of two genes, highly homologous to *nitroporin,* occurred. Similarly, it was found that dsRNA targeting vacuolar H^+ ATPase in Colorado potato beetle (CPB) can also downregulate the orthologous gene in *Diabrotica virgifera.* Hence, for efficient RNAi in *Diabrotica virgifera*, more concentration of dsRNA is required as compared to CPB (Baum et al. 2007).

Moreover, it is not well understood regarding which region of gene (5′ end or 3′ end coding region) is ideal for the design of dsRNA. No difference in mortality in case of pea aphid *A. pisum* was seen in insect groups fed with dsRNA that matched 3′ end or 5′ end of *hunchback* (*hb*) gene (Mao and Zeng 2012; reviewed in Andrade and Hunter 2016). Nonetheless, researchers agree that several dsRNAs need to be screened for a certain gene, and dsRNA needs to be designed in a highly specific manner when it comes to the target gene and insect species. Alternatively, dsRNA should be designed in a way to have broader spectrum toward species which are closely related (Andrade and Hunter 2016).

17.8 dsRNA Length and Concentration

The uptake of dsRNA and efficient downregulation of the targeted gene in unicellular and multicellular organisms depends on the fragment size of dsRNA (Mao et al. 2007; Saleh et al. 2006). According to Kumar et al. (2009), the length of dsRNA for oral ingestion analyses should be among 300–520 bp. Hence, due to the persistence effects of silencing, the smallest suggested length of dsRNA is 211 bp (Saleh et al. 2006). Moreover, it was found that the silencing effect in aphids and aquaporin remains for five days (Shakesby et al. 2009).

Perhaps, dsRNA concentration in the optimal amount has to be determined to induce optimum silencing of the targeted gene. However, it is not correct to say that increasing the optimum concentration of dsRNA enhances silencing efficiency (Meyering-Vos and Muller 2007; Shakesby et al. 2009).

Upon injection of multiple dsRNAs, a competition between dsRNAs well as over-saturation can occur. This may result in poor RNAi response (Miller et al. 2012). Miller et al. (2012) conducted a study in which 30 bp and 60 bp dsRNA induced 30 and 70% of knockdown of gene, respectively. Nonetheless, many studies have pointed out that dsRNAs, ranging between the lengths of 140 bp and 500 bp, is imperative in success of RNAi technology. Some studies have even reported 1842 bp dsRNAs to achieve success in RNAi technology (reviewed in Huvenne and Smagghe 2010). In a recent paper, it has been described that more than 200 bp long dsRNAs are capable of resulting in several siRNAs after the cleavage of dicer. This has contribution in RNAi response and in precluding resistance that arises from polymorphic variation as encoded by the sequence of nucleotides (reviewed in Andrade and Hunter 2016). It is imperative to note that RNAi induction via dsRNA can occur through

siRNAs, engendering yield of siRNAs in a wide range. This may affect the potential for off-target effects. dsRNA processing doesn't occur at fixed/phased 21 bp intervals (Nandety et al. 2015).

17.9 Life Stages of Targeted Insect

To control targeted insect pests in their older phases of life can be more efficient. Conversely, younger life phases of the targeted organism frequently showed better results during silencing. dsRNA targeting *nitropin2* of *Rhodnius prolixus* showed no silencing effect when treated on its fourth instars; whereas, using second instars showed 42% downregulation of targeted gene (Araujo et al. 2006). Similarly, Griebler et al. (2008) observed more silencing effect in fifth instar larvae of the fall armyworm (*Spodoptera frugiperda*) when compared to the adult moths.

In a study conducted by Naqqash et al. (2019), the research showed that the mortality rate engendered by dsRNAs differed in CPB larval stages. In addition, high mortality (90.9–100.0 and 37.6–67.4%) in first and second instar was observed. In case of third instar, the mortality rate of 15.3–50.6% was observed and 1.1–12.9% in case of fourth instar larvae. The results were in line with the study reported by Amiri and Bakhsh (2019) and Hussain et al. (2019), in which they observed earlier CPB instars to be more susceptible than the latter ones. This could be due to the gene co-regulation in the latter stages, which has been reported by Togawa et al. (2008) and Cornman et al. (2008)—also suggesting some genes to be stage-specific. The genes which are closely related could have replaced target gene function at third and fourth instar larvae. This might have led to decrease in mortalities at these stages, which was calculated using identity matrix of P450 and GSS.

17.10 Target Gene

For an efficient RNAi system, the selection of the targeted gene plays an important role. For example, the targeted gene could be related to insect growth, development and may have a primary effect on the survival of insects (such as selecting *v-ATPase* gene as a target for knockdown can cause higher mortality ratio). Likewise, targeting a gene for silencing, which has a secondary effect on insect survival and development, may not cause a higher mortality rate because of genetic redundancy (Zhu et al. 2011; Kaplanoglu et al. 2017).

17.11 Ingestion of dsRNAs Potential for Pest Management

For many years, the idea of dsRNAs ingestion has been recognized as a route of administration with a huge potential in the management of pest (Scott et al. 2013). Firstly, insect-fed long dsRNAs can be consumed by midgut cells. Furthermore, siRNAs are generated from these long dsRNAs through insect's dicer endoribonuclease (Baum et al. 2007; Bolognesi et al. 2012). Gene silencing by RNAi can be possible if the fed dsRNA sequence matches with the insect gene, disrupting its expression (Bolognesi et al. 2012). The reasons that make this approach successful for control of insect population are: sufficient risk to develop resistance due to silencing of insect genes, and specificity and efficiency in knocking down targeted genes of sucking or chewing insect via transgenic or sprayable delivery methods (Denecke et al. 2018).

dsRNA can be expanded into an insecticide that can be specific for a certain species by aiming vital insect-pest genes (Whyard et al. 2009). Transgenic plants expressing dsRNA targeting damaging insect-pest genes impaired insect development and were proven to be a successful strategy in plant protection (Mao et al. 2007; Zhu et al. 2012). For efficient uptake of dsRNAs in target insect, the size of dsRNA should be at least 60 bp to achieve effective biological activity inside insect gut (Bolognesi et al. 2012). However, the accumulation of the extreme amount of long dsRNA prevented plants' endogenous system to generate small RNAs (Vazquez et al. 2010).

17.12 dsRNA Inside Insect Gut

In many previous insect-pest management studies, the oral delivery method, transgenic plant generation, nanoparticle-based strategy, and sprayable method are recognized to introduce dsRNA inside insect gut.

The insect gut has three parts: foregut, midgut, and hindgut. Among these guts, midgut has more potential to absorb nutrients because of its larger structure and surface area. Furthermore, midgut comprises of three different kinds of epithelial cells: enterocyte cells (columnar) with microvilli specific for nutrients, stem cells, and endocrine cells. These three cells of midgut are responsible for dsRNA uptake and processing of dsRNA (Walski et al. 2017).

From the gut lumen, gut epithelial cells can undergo dsRNA internalization by two pathways as reported-transmembrane channel (Sid-1 like) protein-mediated uptake pathway and the second is receptor-mediated clathrin-depended endocytic pathway. The involvement of both pathways in insects for the uptake of dsRNA and then movement into the hemolymph has been reported. According to several studies, the best-documented pathway is the clathrin-dependent endocytic pathway (for further information, refer to Kunte et al. 2019). In several studies, it has been described through

in vitro and *in vivo* assays that pharmacological inhibition of clathrin-depended endocytic pathway in the gut epithelial cell, especially CPB (Coleopterans), showed a decrease in uptake of dsRNAs (Cappelle et al. 2016).

17.13 Transformative Versus Non-transformative RNAi

The transformative-RNAi for field application consists of transgenic plants or RNAi-based plant characters, while non-transformative RNAi comprises of dsRNAs with end-use products, formulation of dsRNAs, sprayable RNAi, etc.

17.14 Transformative RNAi

Plant-mediated RNAi strategy, also called host-induced gene silencing, for the defense of crops from their specific insect pests are shown to be effective in controlling damaging pests (Baum and Roberts 2014; Baum et al. 2007; Head et al. 2017). Therefore, such a strategy to develop a transgenic plant expressing dsRNA (species-specific) is an effective way of RNAi for plant protection (Joga et al. 2016; Zotti et al. 2018). Plant-mediated RNAi for harmful insect-pest management has proved to be an eco-friendly approach. Furthermore, RNAi is a specific and stable approach to efficiently silence the desired genes (Baum et al. 2007). A specific selection of important genes (target) is the main reason for the efficient RNAi approach (Terenius et al. 2011). Plant-mediated RNAi (transgenic) approach has been potentially developed as a recent contemporary insect-pest control strategy. Expressing dsRNA in the transgenic plants against the targeted gene of specific insects downregulate expression when exposed to transgenic plants (Price and Gatehouse 2008; Mutti et al. 2006, 2008) (Fig. 17.2).

Yu et al. (2014) revealed that a prospective target ecdysone receptor is silenced using RNAi in *Nilaparvata lugens* to make rice plant resistant to damaging insect-pest. Two genes of *N. lugens*, i.e., *NlEcR A* and *NlEcR-B* were selected for this study. They generated 360 bp fragment, *dsNlEcR-c*, orally delivered in their experiment. This significantly downregulated the relative expression of targeted genes as compared to GFP control. Additionally, the fecundity of *N. lugens* was significantly reduced when dsRNA was delivered orally. Therefore, using *Agrobacterium*-mediated transformation, transgenic rice lines expressing dsRNA against *NlEcR* were developed. qRT-PCR and Northern blot analysis confirmed transgenic rice lines expressing *dsNlEcR-c*. Feeding assay was performed by letting neonates of *N. lugens* feed on transgenic plants producing the desired dsRNAs. The insects fed on transgenic rice lines showed significant downregulation of targeted transcript as compared to control. However, the nymphs exposed to dsRNA showed a lower survival rate and a significant decrease in fertility as compared to control (44.18–66.27%).

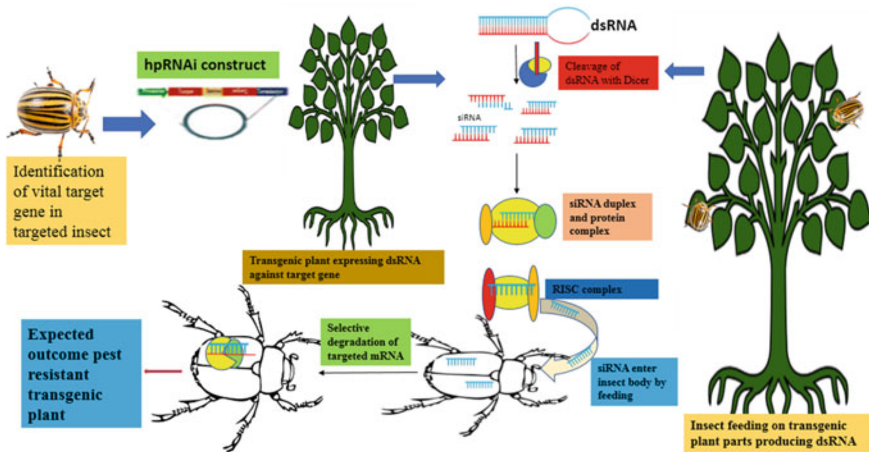

Fig. 17.2 Transformative RNAi. Transgenic plant expressing dsRNA against targeted insect-pest gene (Modified from Mamta and Rajam 2017)

Wild and cultivated tobacco are attacked by specialist herbivores which are closely related: *Manduca quinquemaculata* and *M. sexta* (Lepidoptera, Sphingidae) (Poreddy et al. 2017). Poreddy et al. (2017) reported insect-pest management strategy using plant-mediated RNAi. Mainly, two vital genes (midgut genes of *M. sexta*: *CYP6B46* and *irBG1*) were targeted using dsRNA. The dsRNA was expressed in *Nicotiana attenuata* using *Agrobacterium*-mediated transformation. *M. quinquemaculata* larvae fed on transgenic plants showed significantly reduced transcript level (90%) in the case of *CYP6B46* and 80% in the case of *irBG1*. Moreover, similar results were also observed when tomato hornworm was fed on dsRNA expressing transgenic plants against *irCYP6B46* and *irBG1*. Hence, RNAi-mediated strategy can be an efficient strategy for controlling closely related insect pests.

Ibrahim et al. (2017) reported plant-mediated RNAi insect management strategy by developing dsRNA against the v-ATPase transcript of whitefly. *Agrobacterium*-mediated transformation method was used to transform a lettuce plant using EHA105 strain. Insect feeding assays showed the highest mortality rate of 83.8–98.1% as compared to control lines. The reduction in fecundity was also observed. Furthermore, qRT-PCR analysis revealed decreased transcript level in whiteflies fed on transgenic lines. Such a plant-mediated RNAi strategy can also be used to study insect-plant interaction.

Niu et al. (2017) developed a transgenic plant expressing dsRNA against two important genes: *dvvgr* and *dvbol* of western corn rootworm. Both genes, *dvvgr* and *dvbol,* have an imperative function in the development and reproduction of *Diabrotica virgifera virgifera.* The main aim of this study was to make transgenic maize resistant to western corn rootworm by reducing fecundity. Results revealed that insect

larvae also showed a significant level of reduction in fecundity when fed on transgenic plants. Furthermore, feeding bioassay of adults with different concentrations of artificial diet showed reduced mortality, ranging between 46.5 and 75.5%.

Guo et al. (2018b) developed a plant-mediated RNAi strategy to control *L. decemlineata.* A hairpin type dsRNA targeting acid methyltransferase, a juvenile hormone, was established via *Agrobacterium*-mediated transformation in potato plant. CPB larvae that fed on transgenic lines (foliage part) expressing *dsJHAMT* showed significant reduced transcript levels of the targeted gene. Exposure to *dsJHAMT* showed a negative effect on CPB development and growth, particularly less oviposition. The transgenic lines were shielded from the damage of insects as the insects those survived laid less or no eggs. These transgenic plants expressing *dsJHAMT,* proved to have potentiality in pest control practices.

Hussain et al. (2019) developed a plant-mediated RNAi (transgenic) strategy for the control of serious potato pest CPB. Conserved regions of ecdysone receptor (EcR) were selected and cDNA was amplified and cloned into pRNAi-GG vector (intronic sequence, *pdk*, as a flanking region) for the generation of dsRNA. *Ecr* is a highly specific molting-associated gene having a potential role in insect development and growth. *Agrobacterium* strain LBA4404 was used to transform construct harboring the desired dsRNA driven by *CaMV35S* promoter to develop transgenic potato lines (cv. Agria and cv. Lady Olympia). Transgenic potato cultivars were assessed for their silencing efficiency against CPB first, second, and third instar larvae. A significant level of mortality (15–80%) was observed. Moreover, reduction in larvae weight was detected in transgenic line-fed insects, as evaluated against the control. Furthermore, a significant level of downregulation of *EcR* transcript was also detected in potato transgenic line-fed insects expressing dsRNA. Subsequently, this strategy has the potentiality to manage potato pests.

17.15 Non-transformative RNAi

Spray of dsRNA can activate RNAi-pathway in the desired pest without changing the plant genome. The sprayable RNAi is a non-transgenic method for crop protection and has already been proved to be successful (Hunter et al. 2012). dsRNA delivered through spraying on leaves or through injection in trunks can travel via vessels of the plants to distal distances and these approaches can be considered in pest control approaches (Andrade and Hunter 2016; Hunter et al. 2012). RNAi-based sprayable products can be categorized as growth enhancers, developmental disrupters, direct control agents, and resistance repressors (EPA 2014). According to Li et al. (2015), spraying of dsRNA cannot be an effective tool under the large field condition for all

insect pests, but it can be more effective at a smaller level to control few pests under the field conditions.

Yan et al. (2019) recently reported a novel RNAi-based pest management strategy to control soybean aphid using nanocarrier-based dsRNA delivery system. Previously, they also published a study in which they used detergent and nanocarrier-based transdermal dsRNA delivery methods to evaluate RNAi efficiency in *Aphis glycines*. However, in the current study, they improved dsRNA transdermal delivery by using smaller and cheaper nanocarrier to apply RNA pesticide through spraying dsRNA nanocarrier formation on aphid-infested soybean seedlings. Four vital target genes of *Aphis glycines* were selected (*ATPD*, *ATPE, CHS1,* and *TREH*) and cloned to synthesize dsRNA fragment. These four dsRNA fragments were delivered via the transdermal delivery system and tested for their targeted gene silencing efficiency. A significant level of knockdown of targeted gene expression was observed (ranging from 86.86 to 58.87%). Topical and spray application of four constructed dsRNA fragments resulted in the highest mortality rate; dsAPD and dsATPE (using the topical application) caused 81.67% mortality, while the sprayable application of dsATPD and dsCHS1 caused 78.50% of mortality. Results showed that through topical application of dsRNA via nanocarrier, aphid body wall penetration by dsRNA can occur within four hours. This can be a powerful strategy to practice RNAi-based pest management in the field trials.

Naqqash et al. (2019) recently developed an RNAi-based pest management strategy to reduce the resistance of chemical pesticide (imidacloprid) in CPB. CPB is an important pest of potato crop worldwide and has developed resistance to more than fifty active compounds of different groups of chemicals. Three important genes were selected to downregulate their gene expression under laboratory conditions that belong to cytochrome P450 monooxygenase, cuticular protein, and glutathione synthetase families which encode for imidacloprid resistance in *Leptinotarsa decemlinaeta* (CPB). Fragments of dsRNA-CP, dsRNA P450, and dsRNA-GSS were synthesized and tested by feeding bio-assays of *HT115* expressing dsRNA on leaflets of potato in different phases of imidacloprid resistant CPB lab population. Correspondingly, feeding bioassay results showed decreased insect survival rate exposed to CP-dsRNA: 4.23% in second instar larvae, 15.23% in third instar larvae, and 47.35% in fourth instar larvae. Furthermore, due to dsRNA feeding, pre-adult duration, and larval weight were also disturbed. 100% mortality was observed in second instar larvae when exposed to imidacloprid along with reduced doses of CP-dsRNA and GSS dsRNA. Hence, this novel strategy can be used in the future for the management of CPB populations resistant to imidacloprid (Table 17.1).

Table 17.1 dsRNA delivery via oral feeding (sparayable) targeting vital genes of insects to protect plants

Insects	Target gene	Aim of study	Application	Results	References
Leptinotarsa decemlineata	*COPβ* *Sec23* *vATPases*	Ingestion of dsRNA to knockdown target genes for control of CPB population	Oral feeding	Significant level of mortality, reduction in body weight	Zhu et al. (2011)
CPB	*Shd*	Down-regulation of *LdShd* gene in *L.decemlineata* (CPB) via RNAi approach	Oral feeding	Delayed growth, Significant level of kock-down	Kong et al. (2014)
Leptinotarsa decemlineata	*dsRNase1* *dsRNase2*	Enhancement of RNAi efficiency through silencing of nuclease genes in the gut of CPB	Oral feeding	Significantly silenced and decreased transcript level 84-86%	Spit et al. (2017)
Leptinotarsadecemlineata	RyRs	Using RNAi approach knockdown of RyRs gene in CPB	Oral feeding	Significant level of downregulation of gene	Wan et al. (2014)
CPB	*Actin*	The next generation of insecticides: dsRNA is stable as a foliar-applied insecticide	Sprayable	100% mortality	San Miguel and Scott (2016)
CPB	CP, (GSS, P450)	Downregulation of imidacloprid- resistant genes impacts CPB the biological parameters	Sprayable	47.35% silencing efficiency	Naqqash et al. (2019)

17.16 Use of CRISPR Technologies in Understanding Plant-Insect Interactions

17.16.1 Introduction to CRISPR

CRISPR technology has emerged as a breakthrough technology in plants, animals, and other living cells and organisms to generate targeted mutagenesis. Historically, creating mutagenesis at the target loci specifically posed a challenge in many different species. To prompt genetic changes, double-strand breaks (DSBs) generation is inevitable with the help of sequence-specific nucleases (SSNs) at the intended site in the genome. DSBs repair is mostly mediated by non-homologous end joining (NHEJ) and brings about insertion and deletions (indels) in the genome (reviewed in Sattar et al. 2019; Zhang et al. 2019).

Earlier, two different approaches were being used for gene editing, namely transcription activator-like effector nucleases (TALENs) and zinc-finger nucleases (ZFNs). Two of these technologies make use of the interaction between DNA and protein. TALENs and ZFNs are quite tedious in terms of engineering and multiplexing and were soon replaced with the discovery of programmable CRISPR-based gene editing technology, as CRISPR is a simple, convenient, versatile, and inexpensive system (reviewed in Zhang et al. 2019). After the discovery of CRISPR as an immune system against viruses in bacteria, it has been extensively used in gene editing (GE), with initial utilization of CRISPR in plants been reported in 2013 (Feng et al. 2013; Li et al. 2013; Nekrasov et al. 2013; Shan et al. 2013; Xie and Yang 2013). Frequently utilized nuclease SpCas9 derived from *Streptococcus pyogenes* induces DSBs generated near the protospacer adjacent motif (PAM) sequence (3–4 bps ahead of PAM) with its RuvC and HNH domains. This happens in association with 20-nt seed sequence crRNA (CRISPR RNA) and trans-activating crRNA (tracrRNA). DSB ends may vary, such as in ZFNs an overhang of staggered 4–5 nt cut is produced at its 5′ site, TALENs induce staggered heterogeneous overhangs and SpCas9 produces staggered cut of 1 bp (reviewed in Dangol et al. 2019).

DSB repair is mediated by non-homologous end joining (NHEJ) pathway which is prone to error and inserts or deletes small nucleotides, thereby knocking out the targeted gene (Fig. 17.3). Another repair mechanism homologous recombination (HR) can also work in the presence of a homologous template donor. In the somatic cells of the plants, the NHEJ mechanism is usually predominant over HDR. In the cell cycle, at the G1 phase, NHEJ initiates, which is enhanced with Shielden complex (after Ku protein complex attaches to exposed DNA ends). The broken DNA is made stable by DNA-PKcs. Artemis nuclease complex is recruited for the DNA end processing, followed by ligation of DNA ends via DNA ligase IV for repair, with the incorporation of several bases long mutations. Other mechanisms have also been reported to occur for repair mechanisms such as microhomology-mediated end joining (MMEJ) or alternative end-joining (A-EJ). This depends on microhomology of 5–25 bp to create indels resulting in 3′ overhangs. MRN complex, formed by the association of Nbs, Mre11, and Rad50 proteins, removes the blockages formed at

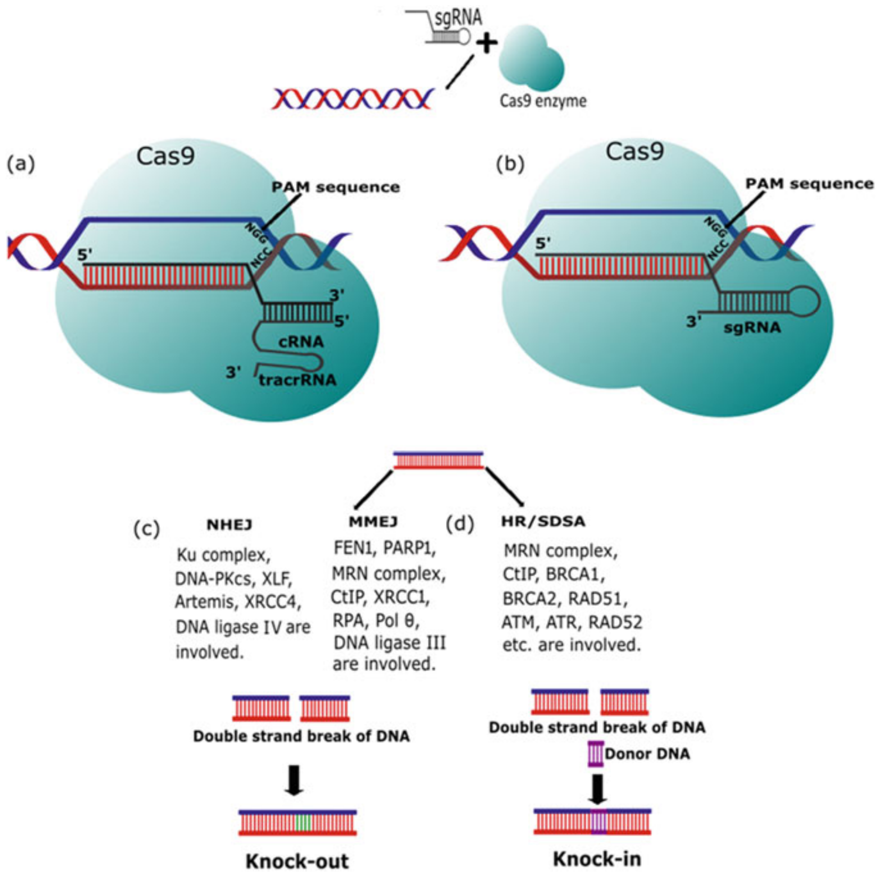

Fig. 17.3 Mechanism of action of CRISPR/Cas9 system. **a** In naturally occurring bacterial system. **b** Artificially synthesized gene editing mechanism. Various molecules and mechanisms involved in: **c** Gene knock-out and **d** Gene knock-in. Double strand break is mediated by ZFNs, TALENs and CRISPR as follows: an overhang of 4–5 nt as well as 5' end staggered cut in case of ZFNs, staggered and heterogenous overhangs in case of TALENs, and a staggered cut of a one-bp for SpCas9 which was also thought to generate a DSB which is blunt-ended. For Cpf1, a staggered cut in the 5' end can be generated (Reproduced from Bortesi and Fischer 2015; Reproduced from Dangol et al. 2019; Zuo and Liu 2016; Guha et al. 2017; Cubbon et al. 2018)

DSB via C-terminal binding protein interacting protein (CtIP). HR-repair may be associated with strand invasion catalyzed by accessory/regulatory proteins as well as RecA, Rad51, or RadA (recombinases). RecA and RecBCD are the pathways being involved in HR-mediated repair by the recombinase. Strand invasion forming displacement loop is repaired by many other pathways, such as repair mediated by DSB, synthesis-dependent strand annealing (SDSA), or break-induced replication (BIR). SSTR (single-stranded template repair), which can be important in gene

editing based on HR mechanism, does not require recombinase enzyme and avoids the formation of D-loop (reviewed in Cubbon et al. 2018; Dangol et al. 2019).

17.16.2 Classification of CRISPR

At the time of defense against virus, if one single multifunctional protein is involved in the stages of processing as well as interference of pre-crRNA, then the CRISPR system can be classified as class 2 system. If not, then it is classified under class 1 system. Every class can further be grouped into classes based on their signatory proteins. Types I, III, and IV are classified under class 1 (Csf1, Cas3, Cas10 are signatory proteins). Class 2 consists of types II (contains Cas9), V (Cas12a, Cas12b, Cas12c, Cas12d, Cas12e, Cas12g, Cas12h, Cas12i, Cas14a, Cas14b, Cas14c), and VI (Cas13a, Cas13b, Cas13c, Cas13d) (reviewed in Zhang et al. 2019).

17.16.3 Utilizing the CRISPR/Cas9 Technology in Modulating Plants-Insects Interactions

Though many insect-pest resistant crop plants have been developed with the RNAi technology, the total silencing of the targeted gene of interest may not be achieved as accomplished by the gene editing technologies. With RNAi technology, complete transcript knockdown may not be achieved and it may be challenged due to competing endogenous gene regulation mediated by RNA and off-targeting is not predictable. CRISPRi may be used in a highly specific and reversible manner in simultaneous repression of several target genes with the prevention of transcriptional elongation and transcription factor or RNA polymerase binding. However, RNAi may be tedious in result interpretation caused by direct/indirect effects of silencing of downstream targets. Since eukaryotes are devoid of CRISPR system, such a competition as seen for RNAi technology is negated (Bisht et al. 2019).

Development of crop resistance to insects can be achieved by CRISPR-based technology, mainly by affecting the target-effector interactions, targeting susceptibility genes of the host, editing the genes related to broad spectrum resistance, eliciting receptors and so on. Direct editing of insect genomes has been performed, especially utilizing the gene drive mechanism and decreasing insects' resistance to insecticides. While performing gene editing in insects and to promote gene drive, knockout of the target in insect genome should be selected, that is lethal or creates an imbalance in sex ratio or insect fecundity. Gene drive is a method where edited genes can be dispersed rapidly in the targeted insect population, in entirety. CRISPR-mediated gene drive can be used to render lethality/sterility in insect populations. Distortion of sex ratio to males may be achieved by exterminating the X-chromosome specifically at the

time of spermatogenesis. Insect pests can be controlled genetically with the release of insect strains harboring these deleterious traits, and the strains carrying viral resistance gene in insect vectors to control viral and vectored diseases. Susceptible and resistance alleles, conferring plant resistance loci, vary by only one or a few bases. The insecticides may be continued to be used in a lesser amount if we could replace the insecticide-resistant alleles with sensitive alleles. Other approaches could be in changing the way target and effector molecules interact, editing susceptible genes related to plant immunity, modulation of gene expression that plays a crucial role in insect feeding, and engineering *R* gene variants synthetically to achieve recognition of insects in a broader range. Additionally, utilization of dCas9 could be an applicable method in future insect-pest management which can activate/suppress insect-pests' insecticide-susceptible or resistant genes (Bisht et al. 2019). To decrease the risk of segregation, site-specific nucleases can be utilized to introduce targeted genes at specific loci and to stack several genes in crop plants. This can also aid in negating undesired footprints such as those in attB/loxP sequences (Bisht et al. 2019).

Genome editing of several insects using CRISPR technology has been successfully achieved in the last few years. Several studies of using CRISPR/Cas9 technology for genome editing of insects have been reported. Many experiments have been conducted in the model insects such as *Bombyx mori*, *Drosophila melanogaster,* and other crop-damaging insects. The ease of designing of a CRISPR construct, helps to elucidate the function of genes (Zhang et al. 2014). So, this fascinating technology is not only limited to unravel the functions of genes, but also has emerged as a game-changer tool for the control of pest insects, and diseases (Reid and O'Brochta 2016). Transgenic crops harboring the genes of *Bacillus thiuringiensis* and insecticides are broadly used for the protection of crops against harmful pests (Bravo et al. 2011). Nevertheless, the progress of developing tolerance against Bt toxins has been increasing, showing an alarming situation toward pest protection.

A cadherin-like receptor of *Bt* Cry1A toxin has been discovered in numerous insects of Lepidoptera (Wu 2014). Several experiments of RNAi show that in several Lepidoptera, cadherin is engaged in Cry1Ac resistance. Wang et al. (2016) injected an amalgam of mRNA of Cas9 and sgRNA into eggs of *Helicoverpa armigera* (cotton bollworm) and targeted the ninth exon of cadherin gene. This experiment showed that the mutated insect cadherin gene exhibited higher resistance compared to the control strain. The obtained results show that the cadherin gene is an essential receptor of Cry1Ac, showing its relation in Cry1Ac resistance (Wang et al. 2016). In two insect pests of lepidopteran order (*Helicoverpa punctigera* and *Helicoverpa armigera*), elevated levels of tolerance to Bt toxin, Cry2Ab, are connected with defunct *ABC* transporter gene (*ABCA2*). To prove the association between Cry2Ab and ABCA2 (*HaABCA2*) in *Helicoverpa armigera*, two different knockout lines, *HaABCA2* of SCD strain were generated using CRISPR/Cas9 method. A high level of resistance to both Cry2Ab (greater than 100-fold) and Cry2Aa (greater than 120-fold) was observed in both knockout strains. However, very limited resistance or no resistance was obtained to Cry1Ac (less than fourfold). In their study, they showed

that *HaABCA2* can have an imperative function in the mediation of toxicity in either of the toxins (Cry2Aa and Cry2Ab) against *H. armigera* (Wang et al. 2017).

CRISPR/Cas9 technology has been used in editing the pigment genes of *H. armigera* and these mutations showed several physical phenotypical changes (Khan et al. 2017). Chang et al. (2017) conducted a study to abolish the mating in *H. armigera* with the help of genome editing of insects (Chang et al. 2017). In another study, two single guide RNAs (gRNAs) were used for the deletion of the cluster of genes. For editing of *CYP6AE12* cluster, Cas9 protein and two sgRNAs were injected into the embryo of *H. armigera.* In a total of 400 eggs injected, 125 eggs hatched. From these hatched eggs, 65 developed into adults. The finding shows that the genome editing of such genes influenced the rate of survival of cotton bollworm (Wang et al. 2018).

Kandul et al. (2019) have demonstrated the adoption of CRISPR technology in developing a sterile insect technique (SIT) known as a precision-guided SIT (pgSIT). SIT has been described as a safer technology in terms of environment corroborated to suppress the wild population of insects. pgSIT technology depends on the dominant genetic technique allowing sexing and sterilization at the same time. gRNAs were designed to target exons of *Drosophila* female-specific and male-specific genes. The engineering of multiple pgSIT systems ensured that the eggs are laid in the environment that would lead to the generation of 100% non-fertile, competitive, and fit adult males. The study used mathematical methods and predicted that pgSIT would suppress a larger population, which has potentiality in controlling disease vectors and agricultural pests.

Lu et al. (2018) have used CRISPR/Cas9 technology by suppressing the serotonin biosynthesis to achieve resistance against two devastating rice pests: stem borers and planthoppers. The insect infestation causes the induction of serotonin biosynthesis in rice. Tryptamine is converted to serotonin via tryptamine 5-hydroxylase catalysis, which is encoded by *CYP71A1* (cytochrome P450 gene). Salicylic acid as well as serotonin biosyntheses are induced upon planthopper infestation in wild-type susceptible rice. Mutated *CYP71A1* gene produced no serotonin with elevated salicylic acid, rendering the plants with ameliorated insect resistance. Serotonin application in mutant rice was shown to have lost its resistance against the insect, and the artificial diet supplemented with serotonin fed to insects led to increased insect performance.

Douris et al. (2016) generated *CHS1* (the *chitin synthase 1*) gene mutant (I1042M) of BPU (benzoylureas)-resistant *Plutella xylostella* at the exact site as the spider mite I1017F mutant conferring etoxazole resistance. The study incorporated both of these substitutions (I1056M/F) on *CHS1* in *Drosophila* using the genome-editing system integrated with the homology-directed repair (HDR) pathway. These strains were sustained on cabbage plants (*Brassica oleracea*). The study is crucial in resistance management strategies, mostly on the rational utilization of important agricultural pests, based on the rotation of insecticide mode of action groups. With the repetitive application of chemistries associated with the same binding site, this strategy will circumvent the target-site resistance selection in pest management.

Some secondary metabolites in plants, known as protease inhibitors (PIs), target gut proteases of insect herbivores. PIs are naturally occurring secondary metabolites used in defense mechanisms in plants that inactivate digestive proteases of insects and finally lead to its digestion. Plant defense genes become active with the help of different signaling molecules during the invasion of insect herbivores, pathogenic stress, and wounding. These signaling molecules function together systemically and locally. Nearly every class of digestive proteases of insects can be targeted by plant protease inhibitors. Overexpression of PIs in plants can help the management of pests. In previous studies, inhibition of development and growth of larvae and increased mortality was observed in insects fed on plants in overexpressing PIs (Singh et al. 2018). The knockdown of inhibitor genes in insects causes insect mortality, such as knocking down inhibitor genes of serine protease via RNAi-mediated inhibition, elevated *Plutella xylostella* mortality when challenged by destruxin A (mytotoxin) (Han et al. 2014). In a study performed by Leplé et al. (1995), *OCI* (*oryzacystatin I*) gene, a gene encoding cysteine proteinase inhibitor in rice, was introduced and expressed in poplar to obtain insect-resistant poplar. In this study, the crucial digestive proteinases of *Chrysomela tremulae* were analysed. The results obtained from feeding trials of *OCI*-expressing poplar leaves showed that the OCI is toxic to *C. tremulae* larvae. CRISPR system may be used in such approaches to directly target the insect digestive proteinases.

At least 260 crop species such as nuts, vegetables, and fruits are infested by the Mediterranean fruit fly (*Ceratitis capitata*), also known as medfly. The medfly is an invasive agricultural pest. CRISPR-Cas9 has been used via ribonucleoproteins (RNPs) pre-assembly, delivered *in vitro* to the early embryos of medfly. The pigmentation gene of the eye (*we*; eye pigmentation is white) has been targeted with a huge somatic mosaicism rate in adults (G0). Large deletions in the *we* gene was observed with the use of two sgRNAs (Meccariello et al. 2017).

Fall armyworm is a dangerous pest of maize which decreases the yield of maize. Recently Wu et al. (2018) reported effective insect genome editing with the help of CRISPR (indel mutations) in the *abd-A* gene of fall armyworm. Most of the mutant moths were found to be sterile for the single gRNA (targeting *abd-A* gene), injected into embryos of fall armyworm, out of a total of 244 embryos studied. CRISPR/Cas9 technology was successfully used for editing the genome of fall armyworm insects due to its high efficiency. Such an approach can help in developing modified and enhanced resistance against harmful insects of crops (Fu et al. 2013; Pattanayak et al. 2013) (Table 17.2).

Table 17.2 Selected examples of gene editing studies conducted in insects

Insect	Delivery	Target gene	Cells	Editing	Transformation method	Reference
Anopheles gambiae	Plasmid	*AgAP005958, AgAP011377, AgAP007280*	Germline	Knock-out, Knock-in	Embryo microinjection	Hammond et al. (2016)
Drosophila suzukii	Plasmid	*w (white)*and *Sxl (Sex lethet alal)* gene	Germline, Somatic	Knock-out	Embryo injection	Li and Scott (2016)
Aedes aegypti	mRNA	*Nix* (Maledeterming factor)	Somatic	Knock-out	Embryo injection	Hall et al. (2015)
Spodoptera litura	mRNA	*SlitPBP3*	Germline	Knock-out	Microinjection	Zhu et al. (2016)
Spodoptera litura	mRNA	*Slabd-A(S. litura Abdominal-A)*	Somatic	Knock-out	Embryo injection	Bi et al. (2016)
Spodoptera littoralis	mRNA	*Orco* (olfactory receptor co-receptor)	Germline	Knock-out	Egg injection	Koutroumpa et al. (2016)
Helicoverpa armigera	mRNA	*HaCad(cadherin gene)*	Germline	Knock-out	Embryo microinjection	Wang et al. (2016)
Plutella xylostella	mRNA	*P. xylostella abdominal-A (Pxabd-A)*	Germline	Knock-out	Embryo microinjection	Huang et al. (2016)
Locusta migratoria	mRNA	*Orco* (olfactory receptor co-receptor)	Germline, Somatic	Knock-out	Microinjection	Li Y. et al. (2016)
Tribolium Castaneum	mRNA. Plasmid	enhanced green fluorescent protein (*EGFP*)	Germline, Somatic	Knock-out, Knock-in	Embryo injection	Gilles et al. (2015)

17.17 General Lab Practices in Gene Editing Plants and Insects

The first step in gene editing a plant or insect is to have a target gene of interest sequenced (including all alleles) for a particular variety of plant or insect species. Various online web tools have been expanded in the prediction of sgRNA targeting a gene, such as CRISPRko (https://portals.broadinstitute.org/gpp/public/analysis-tools/sgrna-design); however, comparison of various sgRNAs designed based on the online tool (https://crispr.cos.uni-heidelberg.de/) in potato showed no correlation between predicted efficiencies (Nakayasu et al. 2018; Khromov et al. 2018).

The second step would be to recognize off-target mutations (undesired mutation in other parts of the genome that has sequence similarity with target gene) and eliminate them in advance during gRNA design by the testing activity of gRNAs. Off-targets can happen as Cas9/sgRNA can target non-target genes. Mismatches that occur in 'seed sequence' at the first 8 nucleotides (far away from PAM) may be tolerated by the Cas9/sgRNA complex (Tang et al. 2018). It has been reported that up to three bp mismatches in designing sgRNAs could cause potential off-targets in *Drosophila* (Ren et al. 2014); however, up to five bp mismatches in the sgRNA has been reported (Fu et al. 2013). Hence, careful consideration of potential off-target screening should be done while designing sgRNAs. Many online tools such as CRISPR-P, CRISPR MultiTargeter, and specifically for *Drosophila*, DESKGEN, CRISPR flydesign, flyCRISPR may be used in advance while designing sgRNAs (Bisht et al. 2019). Proof-reading Cas9 variants (synthetic), fCas9, RNPs can be used to reduce off-targets (reviewed in Dangol et al. 2019).

The third step is to clone sgRNA(s) to a CRISPR/Cas vector, such as available from various commercial suppliers or laboratories. *Cas* gene is expressed under RNA polymerase II (Pol II) promoter, whereas gRNA under RNA polymerase III (Pol III). Different promoters for gRNAs have been utilized in different plants such as *Arabidopsis* (*AtU6p*), potato (*StU6p* or U3p) and so on. Dual-Pol II promoters have been used instead of Pol III promoters for elevated transcription levels of gRNAs. Pol II promoters have the advantage of the generation of long transcripts, control of spatial and temporal expression as well as application in multiplexed gRNAs (Belhaj et al. 2013; Wang et al. 2015; Reviewed in Zhang et al. 2019). Codon-optimized *Cas* gene for plants has been developed, driven by constitutive plant promoters like *EFA, CaMV35S*, *UBI*, *CMV*, *LTR* (Belhaj et al. 2013). Ribozyme-based technology of CRISPR is useful (single pol II promoter used for both gRNA and *Cas9*) in *Arabidopsis* as well. Through this, U3p and U6p can be avoided to allow the use of more broad-range promoters and promoters that are tissue-specific (He et al. 2017). In insects to target the inevitable genes specifically in germline and not in somatic cells, promoters such as vasa and nanos may be used to decline the editing efficiency. This will circumvent the generation of biallelic mutation that can engender the lethality of embryos. Less imperative genes may be edited using the U6 promoter to render ameliorated efficiency (Bisht et al. 2019).

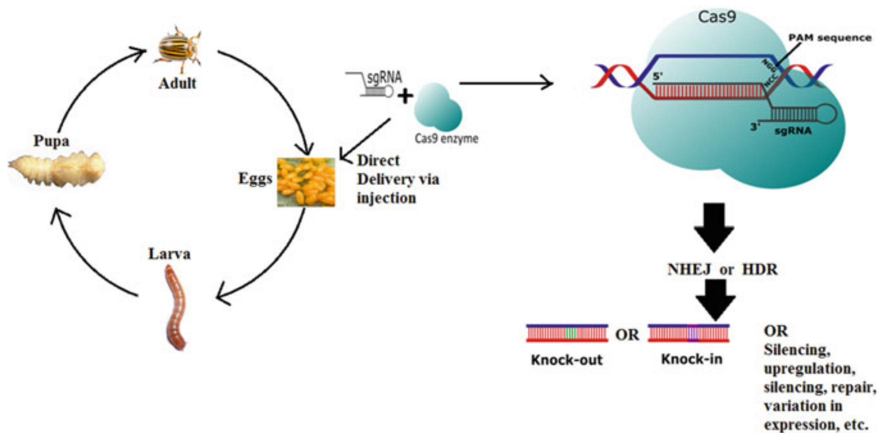

Fig. 17.4 Use of CRISPR/Cas9 system in injecting embryo/eggs for generating gene-edited insects (Modified from Gundersen-Rindal et al. 2017)

The fourth step is to introduce the CRISPR vector system to the plants or insect cells/embryos. Several methods such as viral vectors, transit peptides, biolistics, electroporation, Agroinfiltration, floral dip using *Agrobacterium*, polyethylene glycol (PEG), and *Agrobacterium*-mediated transformations have been used. Besides, the RNP complex has been used in potato transformation using PEG technology (reviewed in Dangol et al. 2019). During the early insect embryo stage, DNA/RNA/proteins can be microinjected (Fig. 17.4); whereas for insect cells, transfection is performed (Bisht et al. 2019; Böttcher et al. 2014). In the case of recalcitrant transfection of cells, a method known as microfluidic membrane deformation has been performed by Han et al. (2015) for the delivery of the gRNA/Cas9 complex. RNA and Cas9 can also be delivered via viral and non-viral vectors to develop transgenic lines/strains, such as transgenic *Drosophila* expressing gRNA and Cas9 has been demonstrated (Bisht et al. 2019; Kondo and Ueda 2013). Nowadays, the central way of delivery of the CRISPR system is microinjecting sgRNA, Cas9, and RNP mixture in case of insect pests as well as several models of animals. This delivery is performed in early cells or even single cells. Applying such a technology in the non-model insects still faces predicaments. Hence, researchers are currently optimizing this procedure in major insect pests that devastate crops globally, such as aphids (hemipteran insects that feed on phloem-sap) (Le Trionnaire et al. 2013). Despite continuous efforts, only a few species of insects have been able to be genome-edited, successfully. These species include locusts, mosquito, some moths, butterfly, and silkworm. The transfer of constructs of CRISPR in the eggs is quite tedious. One group of scientists has introduced a new method in delivering RNP/Cas9 cargo in the germlines of arthropod known as 'receptor-mediated ovary transduction' by injecting female mosquitoes (not eggs) (Chaverra-Rodriguez et al. 2018). The foremost hindrance is the picking up of the insect that has been genome-edited from the brood population. Marker genes like body-color genes or fluorescent protein

genes can be used in screening such population. The assembly of ribozyme-gRNA ribozyme (RGR) can be utilized in one vector harboring several sgRNAs (few for target genes and others involved in the color of the body). Mutant screening can be done easily by visually characterizing the color of the body as well as the fluorescence (Xu et al. 2019). CRISPR-based knockin has failed many times in many lepidopteran insects, especially in silkworm (Lemos et al. 2018). Cpf1 endonuclease has been shown to generate cohesive overhangs following DSBs; such an enzyme is relevant in HDR repair (Zetsche et al. 2015).

The fifth step is to screen mutant plants. Various techniques have been used: Southern blot, genotypic and phenotypic screenings, and PCR amplicon screening (Hua et al. 2017). MSBSP-PCR (Mutation Sites Based Specific Primers-PCR) has been developed for screening of mutants in *Arabidopsis* and *Nicotiana tabacum* for identifying homozygous/biallelic mutations (Guo et al. 2018a). Other PCR based strategies such as restriction enzyme digestion assay, high-resolution fragment analysis (HRFA), as well as polyacrylamide gel electrophoresis (PAGE) have been used. *Cas9* gene presence can be analyzed using RT-PCR. Finally, the actual validation of mutation is done by Sanger sequencing of all alleles of the target gene. This can be achieved by either direct PCR product sequencing or cloning. High throughput or next-generation sequencing (NGS) can be utilized to screen on/off targets, simultaneously (reviewed in Dangol et al. 2019).

17.18 Transgene-Free Approaches

Genetically modified organisms (GMOs) have been widely criticized and backlashed. CRISPR technology can be used to generate non-GMOs to be given a green signal by public and regulatory bodies. T-DNA can be integrated into the cell during *Agrobacterium*-mediated transformation, and must be removed for labeling it as non-GMOs. Transgene-free plants can also be obtained directly, such as Ricroch et al. (2017) obtained transgene-free plants in T2 mutant lines. Protoplast delivery of RNP can be used to forgo intermediates of DNA (Arora and Narula 2017). In avoiding transgenes, the use of FLP/FRT and Cre/loxP piggyback transposons (Zaidi et al. 2018) and suicide transgenes to kill all pollens and embryos at T_0 containing *Cas9* gene have been demonstrated (He et al. 2018). However, CRISPR/Cas13 engineered virus-resistant plants will be required to fall under current GMO regulation as they are transgenic plants (Zhan et al. 2019). Meccariello et al. (2017) developed a DNA-free procedure in generating gene-edited *Ceratitis capitata* without the use of plasmid or transgene for the delivery of Cas9. This may aid in overcoming regulatory laws that preclude releasing GMOs in the environment. Modifying genome utilizing Cas9 can be publicly acceptable with government recognition in such cases. Such an approach can help produce novel and many effective strains in controlling and managing pests (Meccariello et al. 2017).

17.19 Risk Assessment of Release of Gene Edited Insects

Before the release of gene-edited insects, assessments must be undertaken to make sure that unintended targeting of insects does not occur. If the beneficial insects are targeted, then the whole food chain will be affected adversely with negative changes in the community structure. As per the biosafety concerns, terminator genes can be utilized to permit edited insects' programmed life. Also, risk management can be performed using tagged insects which may help trace the gene flow activity (Bisht et al. 2019).

17.20 Use of Other CRISPR/Cas13 System in Insect-Pest Management in Agriculture

Three different Cas13 enzyme systems have been identified as Cas13a, Cas13b, and Cas13c (Cox et al. 2017). Cas13a (C2c2) belongs to class 2 type VI-A system of CRISPR. This was isolated from *Leptotrichia shahii* (LshCas13a). LshCas13a recognizes 22–28 nt long sequence (target) adjacent to PFS (protospacer flanking sequence) of H (A, U, or C). It has been shown that Cas13a is involved in RNase activity processed by RNA guide, a single guide crRNA molecule. Cas13a targets RNAs by cleaving single-stranded RNA molecules (Fig. 17.5a). It has been shown that LwaCas13a is not promiscuous, unlike in prokaryotes, in eukaryotes (including plants). Hence, this is a suitable technology to be used in plants and has been demonstrated in rice protoplasts (LwaCas13a), *N. benthamiana* and *Arabidopsis* (LshCas13a) (Abudayyeh et al. 2016; Aman et al. 2018; Cox et al. 2017; reviewed in Zhang et al. 2019). dCas13 (catalytically inactive Cas13) has been combined with the deaminase domain of human ADAR to generate inosine from adenine, called RNA Editing for Programmable A to I Replacement (REPAIR). REPAIR edits genes by the elimination of mutations related to pathogenicity (Cox et al. 2017).

For agricultural purposes, CRISPR/Cas13 can be utilized in the detection of nucleic acids in plant genes which may be beneficial for various purposes such as breeding, identification of pathogens as well as detection of traits during breeding programs (Abudayyeh et al. 2019). Abudayyeh et al. (2019) performed amalgamation of SHERLOCK in association with Cas13 that has been combined with a fluorescent quenched reporter RNA or RNA lateral flow reporter. The study aimed to focus on soybean research in identifying levels of glyphosate resistance gene as well as the ability to identify multiple plant genes at a given time in a single reaction. Such an approach could be used in identifying the susceptible and resistant genes in insects to pesticides as well as in plants against insect pests.

To gain resistance against the *Turnip mosaic virus* (TuMV) (vectored by minimum eighty-nine aphid species) (Adachi et al. 2018) in *Nicotiana benthamiana*,

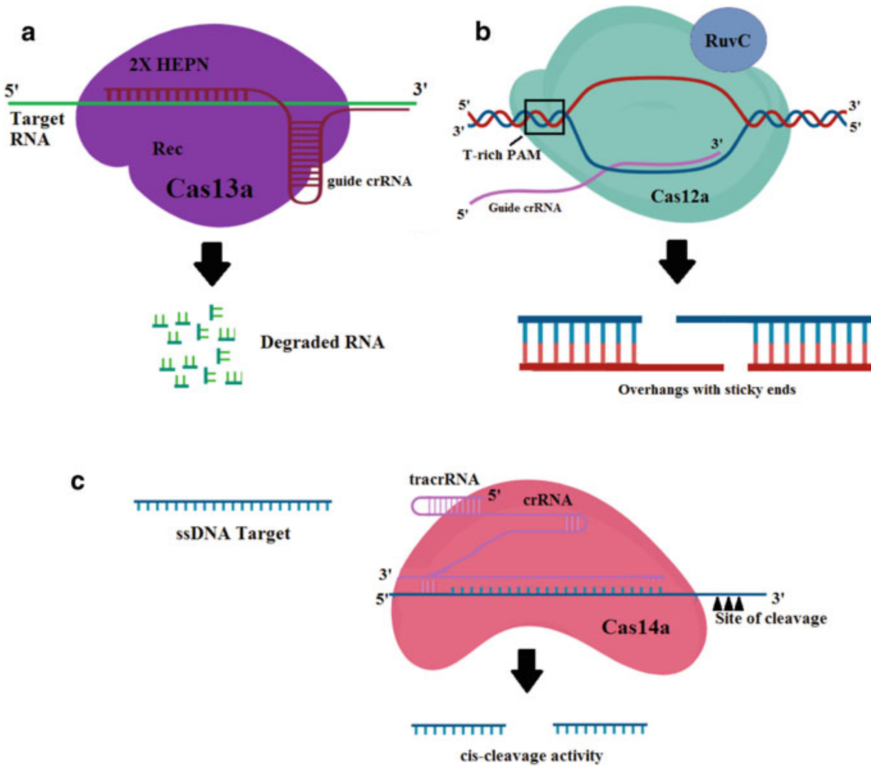

Fig. 17.5 Mechanism of action of various CRISPR technologies. **a** CRISPR/Cas13a, **b** CRISPR/Cas12a and, **c** CRISPR/Cas14a (Modified from Aquino-Jarquin 2019; Reproduced from Zaidi et al. 2017; Liu et al. 2017 and Aman et al. 2018; Modified from Dangol et al. 2019; Schindele et al. 2018)

CRISPR/Cas13a RNAi system has been used. Their data showed that this system can help in interference with the viral genome (Aman et al. 2018). Zhan et al. (2019) designed sgRNAs that target three different PVY strains and tested the CRISPR/Cas13a system to achieve resistance in a broad spectrum to several PVY (*Potato Virus Y*) strains in transgenic potato plants. They also reported that CRISPR/Cas13a did not target other viral genomes like PVS and PVA. CRISPR/Cas13a technology can be similarly utilized in targeting mRNAs of insects in developing resistance in plants against insect pests.

17.21 Future Prospective with Cas3, Cas12, Cas14 Systems and Cas9 Variants in Pest Management

Biallelic mutations have been generated using the CRISPR/Cas12a technology; Cas12a is an endonuclease that has been categorized under the Class II type V system of CRISPR and is also known as Cpf1. Cas12a enzyme is generally extracted from *Prevoltella* and *Francisella1*. This technology has so far been utilized in rice, *Arabidopsis,* tobacco, tomato, citrus, cotton, and soybean. The PAM sequence that is recognized is T-rich (5′ TTTN 3′) in association with one single crRNA at 5′ end (Fig. 17.5b) (Lei et al. 2017; Xu et al. 2017; Reviewed in Dangol et al. 2019; Reviewed in Zhang et al. 2019). Cas12a has been utilized from *Lachnospiraceae bacterium* ND2006 (LbCpf1) and *Acidaminococcus* sp. BV3L6 (AsCpf1); AsCpf1 is more effective in human cells (Kim et al. 2017). In rice, LbCas12a and FnCas12a have been utilized for homology-directed insertion and were much better in the rates of HR generation when compared with SpCas9 (Begemann et al. 2017). Cas12a can be utilized for those genes which have high AT content (reviewed in Dangol et al. 2019). Cas12a variants have been reported in zebrafish and mammalian cells to ameliorate gene editing efficiency with two varying NLS at C-terminus. Utilization of complete direct repeat pre-crRNAs with GC-base substituted stem-loops has been shown to be useful (Liu et al. 2019; Reviewed in Dangol et al. 2019).

So far, there are no reports of Cas12a nucleases, but dCas12a (dead Cas12a) have been obtained, as well as dLbCas12a, dAsCas12a, and ddCas12a (DNase-dead Cas12a). More engineering has been done, such as enAsCas12a, which can recognize different PAM sites with improved activity at lower temperature. Cas12b endonuclease, also known as C2c1, belongs to class 2 type V-B. This endonuclease engenders 5′ overhang of 7-nt DSBs (staggered) and depends on 5′-T-rich sequence of PAM. It needs both tracrRNA as well as crRNA. Both Cas12a and Cas12b consist of NUC and REC lobes but does not contain HNH domain. The orthologues of Cas12b can be used in plants with better activities at lower temperatures. Cas12e (also known as CasX) targets double-stranded DNA, requires 20-nt gRNA and tracrRNA; it is a dual-RNA-guided Cas enzyme recognizing PAM sequence of 5′-TTCN which engenders roughly 10-nt overhang consisting of the staggered end. Deactivated CasX can also be developed (reviewed in Zhang et al. 2019).

CasY has also been identified (also known as Cas12d) which recognizes 5′-TA PAM that cleaves double-stranded DNA. Similarly, Cas3 has been found to generate large deletions as much as 100 Kb and Cas14 can cause cleavage of ssDNA without any PAM sequence requirement. Cas14 may be used in plant ssDNA virus interference (Fig. 17.5c) (reviewed in Zhang et al. 2019). Such approaches may be used in understanding insect-pest management in crops.

nCas9 can be produced (known as Cas9 nickase) by the generation of D10A point mutation in the domain of RuvCI or HNH domain as H840A. This can generate a nick in the DNA. When two of these mutations are generated, then the upshot is dCas9 (dead Cas9) which has no catalytic activity (reviewed in Zhang et al. 2019). These may be applied in the plants to offset off-target mutations in plants

(Dangol et al. 2019). dCas9 is utilized to study gene expression (ectopic regulation) by fusing it with various inhibitors and activators in the domains of transcription factors. dCas9 can be delivered to target genes such as epigenetic marks as well as enzymes for editing modifications of histones and methylation of DNA (Bortesi and Fischer 2015). fCas9 can be generated using dCas9 fused to FokI chimeric protein as dCas9-FokI. Two dCas9-FokI can be associated with each of the two target sgRNAs to produce a double-strand break where two FokI nuclease domains dimerize (Ott de Bruin et al. 2015; Bortesi and Fischer 2015). This has been shown in rice to lower off-target mutation (Mikami et al. 2016). Such technologies may be utilized in the future in manipulating various gene expressions in plants and insects as well as their understanding of gene regulation.

17.22 Programmable Base Editing in Insect-Pest Management

Base editors with the fusion of cytosine deaminase with dCas9 cytosine deaminase have been developed. Adenine base editor (ABE) can convert A to G in the genomic target. The ABEs can be used to efficiently generate point mutations with minimized off-target mutation and correction of mutations that cause diseases (Gaudelli et al. 2017). In rice, APOBEC1-XTEN-Cas9(D10A)-NLS coding sequence has been utilized to direct point mutation from C (cytidine) to U (uridine) in *NRT1.1B* gene to achieve amelioration in efficiency of nitrogen use and *SLR1* gene (that codes for DELLA protein) to reduce the height of the plant (Lu and Zhu 2017). ABE has been further developed to ABE7.10 with tRNA adenosine deaminase linked to nCas9 to generate point mutation from A to G in wheat and rice (Li et al. 2018). *Danaus plxippus*, the monarch butterflies, can co-exist and colonize plants that can generate toxins called a cardiac glycoside. CRISPR-Cas9-based base editing was performed on the *D. melanogaster Atpα* native gene. It was shown that the resulting triple mutant ‘monarch flies’ conferred resistance and was insensitive to cardiac glycoside (Karageorgi et al. 2019). Base editing approach can be used to specifically correct the plant genes from a susceptible ones to resistance ones against the insects, as well as for its direct application in insects.

17.23 Latest Use of Leaper Technology in RNA Editing

Very recently, the use of a new technology called LEAPER (leveraging endogenous ADAR for programmable editing of RNA) has been utilized in multiple human primary cells which make the utilization of short engineered molecules called ADAR-recruiting RNAs (arRNAs). The technology seeks to address the issues faced by current RNA editing technologies regarding immunogenicity, aberrant effector

function, or delivery barrier. Unlike RNAi technology, ADAR1 or ADAR2 native enzymes are recruited by arRNAs to change the specific nucleotide to inosine from adenosine. The study found up to 80% efficiency in editing frequency when delivered via synthetic oligos, viral, or plasmid vector modes. This technology may be adapted in the plants similar to dCas13b fashion in the REPAIR system conducted in mammalian cells for precise RNA editing to correct aberrant pathogenic mutations (Qu et al. 2019). The use of LEAPER is at its preliminary stage; such a technology may be useful in the understanding of plant-insect interactions.

17.24 Conclusions and Perspectives

Various biotic and abiotic stresses damage crop plants and impede plant growth, development, yield and economic losses. RNAi and CRISPR technology have been utilized to silence the key genes of insects that afflict the crop plants. Though RNAi technology has been widely useful in controlling insect pests, specially spraying dsRNA nanocarrier technology is gaining public acceptance, CRISPR technologies hold promises in comprehending plant-insect interaction and to protect the crop plants from insects. CRISPR-Cas9 technology also has added advantages of producing lower off-target effects (Xu et al. 2019). Addtionally, CRISPR-Cas9 system is simple, robust, and convenient in design and vector construction, capable of developing non-transgenics, and for its precision-based knockout of target gene with higher efficiency. In addition, various technologies of CRISPR variants and alternatives have been discovered and developed, which can be promising in future plant-insect interaction applications.

Acknowledgment Mr. Sarbesh Das Dangol is TÜBITAK (The Scientific and Technological Research Council of Turkey) fellow availing Graduate Scholarship Programme for International Students-2215 (Ph.D.). Besides that, the research work on RNA interference technology in the laboratory of corresponding author was supported by TÜBITAK (Grant No. 215O520). Therefore, the support of TÜBITAK is highly acknowledged.

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