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RNA Interference and CRISPR/Cas Gene Editing for Crop Improvement: Paradigm Shift Towards Sustainable Agriculture

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Abstract

With the rapid population growth, there is an urgent need for innovative crop improvement approaches to meet the increasing demand for food. Classical crop improvement approaches involve, however, a backbreaking process that cannot equipoise with increasing crop demand. RNA based approaches i.e. RNAi-mediated gene regulation and site-specific nuclease based CRISPR/Cas9 system for gene editing has made advances in the efficient targeted modification in many crops for the higher yield and resistance to diseases and different stresses. In functional genomics, RNA interference (RNAi) is a propitious gene regulatory approach that plays a significant role in crop improvement by permitting down-regulation of gene expression by small molecules of interfering RNA without affecting the expression of other genes. Gene editing technologies viz. clustered regularly interspaced short palindromic repeat (CRISPR)/CRISPR-associated protein (CRISPR/Cas) have appeared prominently as a powerful tool for precise targeted modification of nearly all crops genome sequence to generate variation and accelerate breeding efforts. In this regard, the review highlights the diverse roles and applications of RNAi and CRISPR/Cas9 system as powerful technologies to improve agronomically important plants to enhance crop yields and increase tolerance to environmental stress (biotic or abiotic). Ultimately, these technologies can prove to be important in view of global food security and sustainable agriculture.

Keywords: Crop, CRISPR/Cas9; Resistance; RNA interference; Stress

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1. Introduction

Food plays a vital role in the existence of human life on earth. With a rapidly growing population, it is, however, very difficult to fulfill the increasing demand for food globally by using traditional methods of crop improvement. People are making continuous efforts to improve crop yield, nutrient content, and to make disease-resistant crops by using conventional methods of crop improvement. Unfortunately, these plant breeding methods are not viable with the current needs of a fast-growing population as these approaches are laborious and time-consuming.

It has been evaluated that by the year 2050, there is an urgent need for increasing food production by 70% to feed the expanding population globally (Godfray et al. 2010). In the modern era of biotechnology, a range of approaches are in use viz. crossbreeding, transgenic breeding and mutation breeding, for the production of genotypes that are disease-resistant and resilient to climate change and other stresses. However, crossbreeding and mutation breeding are untargeted breeding methods with really backbreaking processes as well as production and commercialization process of genotype produced also faces many limitations whereas, in case of transgenic breeding, apart from long and costly commercialization process genetically modified crops also encounter the challenge of public acceptance (Chen et al. 2019).

Recently, many advances have been made in the RNA-based gene regulation approach; i.e., RNA interference (RNAi), a gene regulatory tool which has been significantly diversified for crop improvement by modifying the expression of the gene for better traits quality with fewer biosafety issues as an expression of the transgene is absent in transgenic lines. RNAi is a gene silencing phenomenon, which can be employed for the assessment of gene function, plant metabolic engineering, and in development of stress-tolerant and disease-resistant crops (Pathak and Gogoi 2016).

Over the past five years, RNA-guided nucleases based gene editing approach i.e., clustered regularly interspaced short palindromic repeat (CRISPR)/CRISPR-associated protein (CRISPR/Cas), has recognized to be an efficient tool for targeted gene editing in crops (Jinek et

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al. 2012). For the genome modification, dsDNA breaks are introduced at specific locations by site-specific nucleases which further stimulates DNA repair mechanisms; i.e., non-homologous end joining (NHEJ) and homology-directed repair (HDR) to introduce specific genome modifications. The NHEJ pathway works by ligating the broken ends of DSB without using homologous DNA which results in insertions or deletions (InDels) or single nucleotide polymorphism (SNP) at the cut site leading to frame-shift or nonsense mutations. In the case of HDR, gene replacement takes place with the help of a homologous template at the breakpoint. Therefore, both NHEJ and HDR plays an important role in nuclease based gene editing (Pardo et al. 2009). In crop breeding, this approach generates the transgene-free bred-cultivars. In this regard, this review encompasses various roles and possible applications of RNAi and RNA guided CRISPR/Cas9 system as powerful technologies to improve agronomically important crops to significantly enhance crop yields and tolerance to various environmental stress agents both of biotic or abiotic origin. Limitations, challenges, and potential future development have also been discussed.

2. RNA interference (RNAi)

RNAi is an evolutionarily conserved naturally occurring gene regulatory phenomenon in eukaryotic cells. It has been evolved to protect cells against invading foreign DNA. Besides this, it also helps in maintaining genomic stability, transposon movement regulation, epigenetic modification, and controls cellular processes at transcriptional and translational levels (Mamta and Rajam 2018). Gene silencing phenomenon was unfolded accidentally in *Petunia* flowers when Napoli et al. (1990) were experimenting to deepen the color of petunia flowers by upregulating the gene coding for pigment production, which surprisingly resulted in variegated flowers instead of expected deep purple flowers. Since the expression of a homologous endogenous gene, as well as a transgene, was suppressed, the phenomenon was called “co-suppression” (Napoli et al. 1990). Fire et al. discovered the same phenomenon in the nematode *Caenorhabditis elegans*, when they injected dsRNA in *C. elegans* which resulted in efficient silencing of target endogenous gene homologous to RNA, hence named the phenomenon as RNA interference (RNAi) (Fire et al. 1998). This turned out to be one of the most compelling discoveries in biotechnology, because of its targeted gene regulation, accuracy, and heritability.

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Similar mechanisms have also been observed in fungi as “quelling” (Romano and Macino 1992), bacteria as CRISPR/Cas system (Wilson and Doudna 2013), algae (Cerutti et al. 2011), fruit fly (Hammond et al. 2000) and mammals (Elbashir et al. 2001). Since then research in this field is burgeoning and researchers feel that RNAi is a promising tool for gene regulation with greater potential as compared to other post-transcriptional gene regulation technologies such as antisense technology. RNAi is a naturally occurring phenomenon in eukaryotes with its oldest and omnipresent antiviral defense system whereas, almost all antisense RNAs are found in prokaryotes (Ali et al. 2010).

In this biological process, small non-coding RNAs (21–28 nt. long), which participate in the gene regulation, are the cleavage product of dsRNAs; i.e., micro RNA (miRNA) and small interfering RNA (Si RNA). The process of cleavage is carried out by a multidomain endoribonuclease named DICER or DICER like enzyme which belongs to the RNase III family (Hammond 2005). Finally, these small non-coding RNAs (ncRNA) get associated with RNA-induced silencing complex (RISC), argonaute (AGO) (Hutvagner and Simard 2008), and other effector proteins and make complex that degrades the target messenger RNA (Redfern et al. 2013; Wilson and Doudna 2013). Thus, RNAi can be defined as the capability of endogenous or exogenous dsRNA to inhibit the expression of the gene whose sequence is complementary to dsRNA (Dash et al. 2015).

2.1 RNAi Mechanism

2.1.1 Components of RNAi machinery

Two ribonucleases participate in the RNAi pathway- first DICER and second RNA-induced silencing complex (RISC) where, DICER cleaves the dsRNA into active small non-coding RNAs and initiates the RNAi pathway (Hammond 2005), while RISC with RNase H core enzyme Argonaute (AGO) accomplish the gene silencing (Hutvagner and Simard 2008). Dicer family belongs to the class 3 RNase III enzyme and consists of four domains: N-terminal helicase domain, a PAZ (Piwi/Argonaute/ Zwille) domain, dual RNase III domains, and a dsRNA binding domain. The primary function of these enzymes is to recognize the dsRNA precursor from the RNAi pathway and to generate small non-coding RNA of a specific length (21–24 nt long). Dicer catalysis model proposes that in multidomain dicer enzyme two RNase III domains

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dimerize and form intramolecular pseudo-dimer, which serves as the active center. It has also been suggested that each domain cuts a single strand of dsRNA forming new terminus (Zhang et al. 2004). Finally, the last step of RNAi pathway; i.e., gene silencing by target mRNA degradation is performed by RISC in association with argonaute (AGO) protein and other effector proteins. Argonaute proteins are primarily found in bacteria, archaea, and eukaryotes. The significant function of Argonaute protein is to recognize guide strand termini, cleavage of target mRNA with its nuclease activity, or recruitment of other proteins involved in silencing. RISC with the gene silencing also participates in the cellular surveillance process (Wilson and Doudna 2013) (Ali et al. 2010).

2.1.2 Mechanism of action

Over the last two decades, the functionality of small non-coding RNA in gene regulatory processes of transcriptional gene silencing (TGS) and post-transcriptional gene silencing (PTGS) has continuously been explored. Various classes of small non-coding RNAs have been discovered so far. These include miRNA, siRNA, piRNA (PIWI-interacting RNA), qiRNA (QDE-2-interacting RNA), svRNA (small vault RNA), etc., having different biogenesis pathways and regulatory mechanisms (Aalto and Pasquinelli 2012). Initially, the process of biogenesis of miRNA and siRNA differs to form their corresponding dsRNA precursors as the cellular origin of miRNA is the genomic DNA whereas, siRNA can be generated endogenously via cleavage of dsRNA into smaller segments or it can be exogenously derived directly from the viruses, transposons or transgene. Regardless of these differences, they have similarities in their sizes and sequence-specific inhibitory functions, which clearly suggest that their respective biogenesis pathways and mechanisms are somewhere related to each other. The RNAi pathway comprised four steps: formation of snRNA as a cleavage product of dicer, loading of snRNA into RISC complex, activation of silencing complex, and target mRNA degradation (Ali et al. 2010).

2.2 Micro RNA (miRNA)

miRNAs are 21–24 nucleotide (nt) long small RNAs, which are derived from MIR genes. The biogenesis of miRNA occurs in the nucleus by RNA polymerase II aided transcription of MIR genes, forming primary miRNA (pri-miRNA) transcript of about 1000 nt (Fig. 1). Due to the presence of intramolecular sequence complementarity in pri-miRNA, an imperfect folded-back

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stem-loop or hairpin structure formation takes place which is further processed into short stem-loop precursor known as pre-miRNA with the aid of DCL1 assisted by dsRNA binding protein DRB1or HYL1(Kurihara et al. 2006). This pre-miRNA is again cropped by DCL1 in the nucleus and generates RNA duplex (miRNA:miRNA*) consists of mature miRNA (guide strand) and miRNA* (passenger strand) (Kurihara and Watanabe 2004). The 3'-terminals of RNA duplex get methylated by HUA ENHANCER (HEN1) at 2'-O- hydroxyl group to prevent degradation of miRNA:miRNA* (Huang et al. 2009; Li et al. 2005). After methylation, RNA duplex exported to the cytoplasm where mature miRNA is loaded on to the RISC complex having AGO and other effector proteins. This miRNA induced silencing complex (miRISC) base pairs with the complementary target mRNA completely, then AGO protein with its characteristic nuclease activity degrades the target mRNA (Guo et al. 2016). In case if complete base pairing does not occur between miRISC and target mRNA then miRISC inhibits the translation process.

In 2011, Huntzinger and Izaurralde suggested that miRNA mediated down-regulation of gene expression occurs by (1) miRISC mediated inhibition of translational initiation or ribosome subunit joining, premature degradation of the budding polypeptide chain, and increase drop off of ribosome; or (2) inducing deadenylation and destabilization of target mRNA (Huntzinger and Izaurralde 2011). Expression of miRNA is usually witnessed during the phase of plant growth and development, secondary metabolite synthesis, abiotic and biotic stress, etc. Hence, change in expression and biogenesis of these RNAs could lead to the formation of the crop with agronomically valuable characteristics (Pareek et al. 2015).

2.3 Small interfering RNA (siRNA)

Gene silencing through RNAi can be triggered via long dsRNA or short hairpin precursors, which can perfectly base pairs with the gene to be silenced. The introduction of long endogenous dsRNA directly into the cytoplasm or access of transgene, viral intruders, or transposable elements can ignite the RNAi pathway by recruiting the Dicer or Dicer-like enzymes (Mello and Conte 2004). This Dicer enzyme crops these dsRNAs into short 21-24 nt long SiRNA duplexes with 2nt overhangs at 3'OH end and 5' phosphorylated ends (Bernstein et al. 2001; Hamilton and Baulcombe 1999). Thereafter, SiRNA- induced silencing complex (SiRISC) is recruited and degrades the sense strand (has the precisely same sequence as of target mRNA) of SiRNA whereas, the antisense strand of siRNA along with siRISC get loaded on to the target mRNA in a

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sequence-specific manner (Fig. 2). siRISC incorporation with AGO protein and other effector protein leads to the post-transcriptional gene silencing (PTGS) by cleavage of target mRNA or inhibition of translation (Saurabh and Vidyarthi 2019). Aside from this, siRNAs by chromatin regulation can also participate in the co-transcriptional gene silencing. Dicer-independent siRNA genesis has also been reported in *Neurospora, Caenorhabditis elegans, Schizosaccharomyces pombe, and Arabidopsis* (Aoki et al. 2007; Halic and Moazed 2010; Lee et al. 2010; Ye et al. 2017). These dicer-independent siRNAs mostly arise from transposable elements, intergenic elements, and transgenes (Ye et al. 2017).

2.4 Role of RNAi in crop improvement

In the 21st century, one of the major goals is to provide food security and stop the malnutrition across the world, but the factors i.e. abiotic and biotic stresses, anthropogenic effects, climate changes and depletion of natural resources limiting the crop production globally (Godfray et al. 2010; Tester and Langridge 2010). Thus, to overcome these problems, genetic engineering should be used in a way to manipulate the physiology of plants, genome, and proteome. In this context, RNAi has been extensively explored by researchers for improving a range of crop features including stress tolerance, disease resistance, yield enhancement, etc. (Table 1).

2.4.1 Biotic Stress resistance

In plants, biotic stress is caused by living organisms, especially, viruses, bacteria, fungi, insects, arachnids, nematodes, and weeds. These organisms account for about a 40% loss in the overall yield of six major food and cash crops. RNAi technology has opened up new prospects for crop protection against biotic stresses.

2.4.1.1 RNAi mediated virus resistance

Viruses are the leading agents behind the major loss of crop productivity as it is very difficult to control them due to their diverse strategies to multiply and transmit diseases in the host plant. Therefore, pathogen-derived resistance (PDR) has been considered as one of the most efficient approaches to fighting against viral infections in plants. But there is one more approach; i.e., RNA interference that provides broad-spectrum resistance against viral infections by targeting the multiple regions of a viral gene. It relies on the principle of targeted silencing of the viral

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coat proteins (CP). In 1998, first RNAi mediated virus-resistant potato transgenic lines were reported, which were transformed by simultaneous expression of both sense and antisense transcripts of helper-component (HC-Pr) gene and shown complete resistance against *Potato virus Y* (PVY) (Waterhouse et al. 1998). Missiou et al. (2004) developed potato transgenic lines that were highly resistant to three strains of PVY by expressing the dsRNA derived from 3'-terminal end part of viral coat proteins (CP) which has been reported as the highly conserved region of PVY isolates. Over the recent years, many RNAi mediated virus-resistant cultivars by targeting viral coat protein have been produced i.e. *Beet necrotic yellow vein virus* (BNYVV)-resistant tobacco (Andika et al. 2005), *Plum pox virus* (PPV)-resistant *Prunus domestica* and *Nicotiana benthamiana* (Hily et al. 2007) and *Bean golden mosaic virus* (BGMV)-resistant *Phaseolus vulgaris* (Bonfim et al. 2007).

Si-RNA mediated silencing of *African cassava mosaic virus* (ACMV) by targeting the replication-associated protein 1 (AC1) resulted in a 66% decrease in ACMV genomic DNA (Vanitharani et al 2003). In 2009, Vanderschuren et al. performed an experiment in which they developed dose-dependent RNAi mediated transgenic cassava lines resistant to ACMV. Cassava brown streak disease (CBSD) is considered as one of the threats for cassava (*Manihot esculenta)* cultivation in East Africa. In this regard, Patil et al. first developed cassava plants resistant to CBSD and provided protection against two causative organisms belonging to two different species; i.e., *Cassava brown streak* virus (CBSV) and *Cassava brown streak Uganda virus* (CBSUV) by using RNAi construct containing 397- nt from N-terminal end and 491-nt from C-terminal end of the coat protein gene of the viruses (Patil et al. 2011).

Tobacco streak virus (TSV)-resistant transgenic lines of both tobacco and sunflower (*Helianthus annuus L.)* were produced by RNAi technology using a 421 bp long coat protein gene containing both sense and anti-sense coat protein sequences (Pradeep et al. 2012). Rice strip disease caused by *Rice strip virus* (RSV) was successfully suppressed in two RSV susceptible varieties of *Japonica* by RNAi construct consists of CP gene and disease-specific (SP) gene (Zhou et al. 2012). *Soybean mosaic virus* (SMV) resistant transgenic lines of soybean were produced by introducing a hairpin RNAi construct containing the Hc-Pro gene (Kim et al. 2016). Peanut

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(*Arachis hypogaea L.*) plants resistant *to Tobacco streak virus* were developed using hairpin RNA comprising TSV-coat proteins.

Pooggin et al. 2003 demonstrated that DNA of replicating virus can be used as RNAi target. They used this approach in the silencing gene associated with bidirectional promoters and witnessed recovery from infection of the *Mungbean yellow mosaic India virus* (MYMIV) in *Vigna mungo*.

A study conducted on *Nicotiana benthamiana* and cowpea plants to develop resistance against *Bean common mosaic virus* (BCMV) by exogenous application of RNAi construct containing viral coat proteins to protect plants from aphid mediated transmission of BCMV (Worrall et al. 2019). *Rice tungroo bacilliform virus* (RTBV)- and *Rice tungroo spherical virus* (RTSV)-resistant rice cultivars have been developed by using highly conserved *coat protein 3* (*CP3*) gene in RNAi construct. They observed high resistance in rice against tungroo disease as well as the ability to transmit the virus has also been decreased in transgenic lines (Malathi et al. 2019).

2.4.1.2 RNAi mediated bacterial resistance

Bacteria serve as the biggest hurdle in crop production as they are ubiquitous in nature as well as replicates with great speed and causes infection. Hence, it is important to produce bacterial resistant crops. Escobar et al. (2001) conducted a study on *Arabidopsis thaliana* and *Solanum lycopersicum* (tomato) to suppress crown gall disease caused by *Agarobacterium tuminifaciens* through RNAi technology. For this, they designed dsRNA construct homologous to oncogenes iaaM and ipt which are necessary for tumor formation. Katiyar-Agarwal et al. (2006) demonstrated that *Pseudomonas syringae* infection in *Arabidopsis thaliana* induced biogenesis of endogenous si-RNA i.e., nat-SiRNAATGB2. This siRNA has down-regulated the expression of the PPRL gene, which is considered a negative regulator of the RPS2 resistance pathway.

2.4.1.3 RNAi mediated fungal resistance

Research findings suggest that RNAi technology can be used to enhance resistance against fungi in genetically engineered crops. Gene silencing has been studied using homologous transgenes (co-suppression), antisense or dsRNAs in many plant pathogenic fungi such as *Cladosporium*

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fulvum (Hamada and Spanu 1998), *Venturia inaequalis* (Fitzgerald et al. 2004), *Neurospora crassa* (Goldoni et al. 2004) *and Magnaporthe oryzae* (Kadotani et al. 2003). Enhancement of resistance against *Phytophthora parasitica var. nicotianae* has been observed in *Nicotiana tobacum* by downregulation of the GST (glutathione S- transferase) enzyme gene via RNAi (Hernández et al. 2009). Fusarium wilt has been classified among the most destructive diseases of banana, caused by *Fusarium oxysporum* f. sp. *Cubense* (Foc). Banana transgenic lines developed by intron hairpin RNA (ihp-RNA) mediated expression of si-RNA has shown enhancement in resistance against Foc. This was achieved by the downregulation of a vital gene of Foc fungus and confirmed by performing 6-week long bioassays in the greenhouse (Ghag et al. 2014). In 2015, Chen et al. explored the role of RNAi machinery in the causative agent of wheat head blight; i.e., *Fusarium graminearum* by the aid of hpRNA for silencing the target mRNA. They also studied the importance of FgAgo (Argonaute protein) and FgDicer2 in gene silencing.

Agarobacterium-mediated transformation (AMT) of RNAi constructs act as a potent approach for investigating the role of the gene involved in pathogenesis. Transformation of *Fusarium oxysporum* spores using RNAi construct of three MAP Kinase signaling genes (viz. *Fmk1*, *Hof1* and *Pbs2*) via AMT, resulted in reduced invasive growth on tomato fruits, pathogenesis, loss of surface hydrophobicity and hypo-virulence on tomato seedling (Pareek and Rajam 2017).

Functional analysis of the membrane-localized gene GmSnRK1.1 important for soybean resistance against *Phytophthora sojae* has been done by overexpressing the gene and RNAi silencing. Results obtained show that overexpression of gene increase the resistance whereas RNAi mediated silencing leads to a reduction in resistance (Wang et al. 2019).

2.4.1.4 RNAi mediated insects and nematode resistance

Insects and nematodes are capable of causing severe damage to the crops. Some of the most disastrous nematodes are *Meloidogyne* spp., *Heterodera* and *Globodera* spp., *Pratylenchus* spp., *Helicotylenchus* spp., *Radopholus similis, Ditylenchus dipsaci*, *Rotylenchulus reniformis, Xiphinema* spp., and *Aphelenchoides* spp. (Yogindran and Rajam 2015). Gheysen and Vanholme (2007) suggested that the expression of dsRNA target housekeeping gene and parasitism gene of

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root-knot nematodes (RKN) in host plant led to the resistance from nematode infection. Bioengineering of crops expressing dsRNA that targets the RKN parasitism gene could help in providing broad-spectrum resistance to crop against RK N (Huang et al. 2006).

Cyst nematodes considered as the highly evolved sedentary endoparasites that cause great damage to the crops globally. Through host induced RNAi silencing, all four parasitism genes of sugar beet cyst nematode (*Heterodera schachtii*) have been targeted having *Arabidopsis thaliana* as host, resulted in a decrease in the number of female nematodes. No complete resistance was, however, observed but the reduction in developing nematodes ranges from 23–64% in different RNAi lines (Sindhu et al. 2009). Similarly, enhanced resistance against soybean cyst nematode *Heterodera glycines* has been reported by targeting reproduction and fitness-related genes; i.e., *HgY25* and *HgPrp17* in soybean transgenic lines (Tian et al. 2019).

RNAi-mediated root-knot nematode (*Meloidogyne incognita)* resistance was pursued in *Arabidopsis thaliana* for targeting two housekeeping genes, splicing factor and integrase enzymes. Splicing factor and integrase enzyme are important for nematodes as they play a prominent role in RNA metabolism. Hence, their RNAi mediated silencing resulted in a significant decrease in the number of galls, females, and egg masses(Kumar et al. 2017). Tsygankova et al. (2019) examined RNAi mediated invitro resistance in bread wheat (*Triticum aestivum* L.) against parasitic nematode *Heterodera avenae* using bioregulators of microbial origin.

The success of cry toxin from *Bacillus thuringiensis* as an insecticide has led the foundation of RNAi mediated insect resistance in crops. The RNAi technology came into the limelight when two reports regarding insect control came in the scientific community. Mao et al. (2007) developed transgenic lines of *Arabidopsis* and tobacco plants expressing CYP6AE14- specific dsRNA. In cotton worm, this gene confers resistance against gossypol, a polyphenol compound. When cotton bollworm larvae fed on leaves of transgenic lines, they showed sensitivity against gossypol in an artificial diet. Baum et al. (2007) developed transgenic maize lines resistant to western corn rootworm by expressing dsRNA specific to the gene encoding A subunit of V-type ATPase pump. Thus, these results suggested that the RNAi pathway can be exploited to control pests from harming the plants by targeting the significant gene of insects. V-type ATPase

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subunit-A coding genes were also used crucially to develop resistance against the whitefly *(Bemisia tabaci)* population in tobacco plants (Thakur et al. 2014). Likewise, RNAi mediated whitefly (*Bemisia tabaci*) resistant transgenic lines of lettuce (*Lactuca sativa*) targeting V-ATPase transcripts in whitefly, increased mortality rate of insects feeding on transgenic plants from 83.8−98.1% (Ibrahim et al. 2017).

Wang et al. (2013) reported that 3-hydroxy-3-methyl glutaryl coenzymeA reductase (HMG- CoA reductase, HMGR) is a significant enzyme in the insect mevalonate pathway and can be utilized as a potential target to produce insect-resistant cultivars using RNAi. Similarly, the chitinase (HaCHI) gene important for molting in insects can also be used as a target to produce insect-resistant plants. Through host-induced RNAi, *Helicoverpa armigera* resistant transgenic tobacco and tomato plants were developed (Mamta et al. 2016).

2.4.2 Abiotic stress tolerance

Plants in their natural field conditions constantly get exposed to various abiotic factors such as high salinity, variation in temperature, flood, drought, and heavy metals, which hinders proper growth and development in plants. These factors are also one of the major causes behind huge crop losses globally. The changing climatic conditions and rapidly expanding population demand creates an urgent need to develop more stress-tolerant cultivars. Hence, RNA interference technology can be exploited to develop transgenic cultivars that can cope with different abiotic stresses. Functional genomics studies revealed that novel genetic determinants are involved in stress adaptation in plants, which can be employed to attain stress tolerance (Pardo 2010).

Receptor for activated C-kinase 1(RACK-1) is a highly conserved scaffold protein that plays a significant role in plant growth and development. RNAi mediated downregulation of *RACK-1* gene in transgenic rice plants has shown more tolerance to drought dress as compared to the non-transgenic rice plants (Li et al. 2009). Likewise, disruption of rice farnesyltransferase/squalene synthase (SQS) by maize squalene synthase via RNAi, resulted in enhanced drought tolerance at vegetative and reproductive stages (Manavalan et al. 2012).

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Stress tolerance and development in plants are regulated by miRNA also affecting negatively the expression of the post-transcriptional gene. Wang et al. (2011a) examined that miRNAs are involved in the very early stage during seed germination and identified that miRNA-mediated regulation of gene expression is present in maize imbibed seed. Wang et al. (2011b), reported 32 known members of 10 miRNA families and 8 new miRNAs/new members of known miRNA families that were found to be responsive to drought stress by high-throughput sequencing of small RNAs from *Medicago truncatula*. These findings suggest the importance of miRNAs in the response of plants to abiotic stress in general and drought stress in particular.

OsTZF1 is a member of the CCCH-type zinc finger gene family in rice (*Oryza sativa*). Conditions like drought, high-salt stress, and hydrogen peroxide can induce the expression of *OsTZF1*. Expression of *OsTZF1* gene was also induced by abscisic acid, methyl jasmonate, and salicylic acid. *OsTZF1* gene overexpressed transgenic plants showed enhanced tolerance to high salt and drought stresses, whereas transgenic rice plants in *OsTZF1* gene were silenced using RNAi technology has shown less tolerance. This suggests the role played by *OsTZF1*gene in abiotic stress tolerance (Jan et al. 2013).

Dehydrin proteins play a significant role in protecting plants from osmotic damage. Various research results suggest that overexpression of dehydrin gene *WZY2* provides more tolerance to plant against osmotic stress. A study conducted by Yu et al. (2019) suggests that RNAi mediated silencing of *WZY2* gene in *Arabidopsis thaliana* makes plant intolerant to osmotic stress.

2.4.3 Seedless fruit development

Seedless fruits are generally appreciated by the consumers as seedlessness increases the quality of fruit with the enhancement of shelf life. Seedless fruits can be obtained by parthenocarpy, which involves the development of fruit directly from the ovary without fertilization. In eggplant, seedlessness prevents browning and texture reduction of pulp (Pandolfini 2009). Production of seedless fruits can be induced artificially by disrupting the genes involved in the formation process of seed and seed set. The seed formation process is regulated by the phytohormones both temporally and spatially. Generally, seedless fruit obtained by inducing mutation or alteration in phytohormones shows pleiotropic effects i.e. change in taste, reduced

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fruit size, etc. Hence, for the production of parthenocarpic fruits novel methods with more efficiency should be employed (Varoquaux et al. 2000). It has been shown that seed development in fruits limits the yield in cucumber (Denna 1973; Tiedjens 1928) and tomato (Falavigna and Soressi 1987). Thus, the replacement of seed and seed cavities with the edible fruit tissue is highly desirable and appreciated by consumers, breeding companies, and production companies. Auxin response factors (ARFs) encode transcription factors that control auxin-dependent plant developmental processes. ARF7 factor of tomato (*Solanum lycopersicum*) designated as SlARF7 found to be highly expressed in unpollinated mature ovaries. Further research revealed that the expression of slARF7 remains high from the initial period of flower development to the formation of mature flowers and decreases within 48 hours after pollination. RNAi-mediated development of transgenic tomato lines with a downregulated *slARF7* gene resulted in the generation of parthenocarpic fruits (De Jong et al. 2009). Schijlen et al. (2007) developed seedless tomatoes through RNAi mediated suppression of chalcone synthase (CHS) gene, a first gene used in the flavonoid synthesis pathway. Likewise, post-transcriptional gene silencing of *flavonol synthase* (*FLS*) gene, a vital enzyme for flavonols production resulted in the generation of seedless or less-seeded fruits in tobacco (*Nicotiana tobacum* cv *xanthi*) (Mahajan et al. 2011).

Aucsia genes distinctly expressed in auxin biosynthesis parthenocarpic flower buds of tomato. The silencing of these genes by RNA interference resulted in parthenocarpic fruit development in tomato with some other auxin-related phenotypes (Molesini et al. 2009). Takei et al. (2019) isolated and characterized small parthenocarpic fruit and flower (spff) mutant in a tomato cultivar. Linkage analysis and RNAi based silencing of *Solyco4g077010* gene, which encodes receptor-like protein kinase resulted in impaired male sterility with parthenocarpic fruit set development.

2.4.4 Shelf life enhancement

Fruits and vegetables are more vulnerable to spoilage as compared to cereals because of their nature and composition. Despite being one of the leading producers of fruits and vegetables, India faces massive losses due to post-harvest mishandling, spoilage, pest invasion during storage and transportation. Hence, it is essential to augment the shelf-life of fruits and vegetables to minimize the horticultural losses. This can be achieved by delaying the ripening of the fruit by regulating ethylene biosynthesis, ethylene-mediated signaling, and ethylene response elements

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with the aid of RNAi. In contrast to other phytohormones, ethylene is the gaseous hormone that plays a major role in the process of fruit ripening through a cascade of signals. 1-Aminocyclopropane-1-carboxylate (ACC) oxidase is an enzyme that catalyzes the biosynthesis of ethylene from its precursor ACC. Tomato transgenic lines with enhanced shelf life have been developed by RNAi-facilitated suppression of ACC oxidase enzyme (Xiong et al. 2005). Similarly, expression of three homologs of 1-Aminocyclopropane-1-carboxylate synthase (ACS) was suppressed during the period of ripening in tomato fruits, thereby leading to the production of delayed ripening tomato fruits due to inhibition of ethylene production (Gupta et al. 2013). Meli et al. (2010) have identified and targeted two ripening specific N-glycoprotein modified genes, α-mannosidase (α-Man) and β-D-N-acetylhexoaminidase (β-Hex) and their suppression via RNAi resulted in fruit softening with extended shelf life.

SISGR1 gene encodes for STAY GREEN protein, which regulates fruit color development and ripening by altering ethylene signal transduction in tomatoes. Fruit shelf-life found to be extended in the *SISGR1* gene suppressed transgenic tomato lines (Luo et al. 2013). Repression of two banana E class (*SEPALLATA3*) MADS box genes; i.e. *MaMADS1* and *MaMADS2* through RNAi resulted in transgenic bananas (*Musa ascuminata*) having desirable characteristics such as delayed color development, reduced fruit softening, delayed ripening and extended shelf-life (Elitzur et al. 2016). Yang et al. (2017), reported 22 individual pectate lysase genes in tomato, out of which one pectate lysase gene; i.e., *SIPL*, has been found to be expressed dominantly during fruit maturation. RNA interference studies of *SIPL* revealed that it plays a significant role in the enhancement of fruit firmness, pathogen resistance, and prolongation of shelf life.

2.4.5 Male sterile plants development

The development of hybrid cultivars has augmented productivity due to hybrid vigor and improved uniformity. Hybrid production depends on the development of male sterility in one parent to ensure purity in hybrids for further production of hybrid seeds. Several methods involving conventional as well as genetic engineering has been reported for the production of male-sterile plants however, RNAi has turned out to be one of the most efficient tools in the development of male sterile lines by targeting male-specific genes that participate in tapetum and pollen development. In tobacco plants, TA29, a male-specific gene expressed in anthers during

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microspore development has been targeted using RNAi technology, gave rise to transgenic male sterile lines (Nawaz-ul-rehman et al. 2007). Likewise, the downregulation of the Bcp1 gene of *Arabidopsis thaliana* expressed in both diploid tapetum and haploid microspore resulted in the generation of transgenic male-sterile plants (Tehseen et al. 2010). S-adenosylmethionine decarboxylase (SAMDC) is considered a significant enzyme in the biosynthesis of polyamines in tomato plants. Suppression of *SAMDC* gene in the tapetal tissue of tomato plants leads to the development of male sterility (Sinha and Venkat 2013).

Cytoplasmic male sterility is the maternally inherited phenomenon present in plants. Nuclear genes play a crucial role in the rearrangement of mitochondrial DNA, which is found to be associated with the naturally occurring phenomenon of cytoplasmic male sterility during plant development. Suppression of *Msh-1* –a nuclear gene in tobacco and tomato plants– resulted in reproducible mitochondrial DNA rearrangement with male-sterility (Sandhu et al. 2007).

2.4.6 Flower color modification

Floriculture or flower farming is a field of horticulture that deals with flowers and ornamental plant cultivation. Nowadays, the demand for flowers in different colors and patterns has increased for the purpose of decoration and scents. This can be achieved by silencing the pigment encoding genes using RNA interference technology. A cDNA encoding *chalcone isomerase* (*CHI*) gene isolated from petals of *Nicotiana tobacum* was suppressed using RNAi, thus reducing pigmentation and altering flavonoid components in flower petals (Nishihara et al. 2005). Similarly, flower color alteration in liliaceous ornamental *Tricyrtis* sp. has been reported using RNAi construct TrCHS1 targeting *chalcone synthase* (*CHS*) (Kamiishi et al. 2012). RNAi-facilitated suppression of three anthocyanin biosynthetic genes; *chalcone synthase* (*CHS*), *anthocyanidin synthase* (*ANS*), and *flavonoid 3',5'-hydroxyla*se (*F3'5'H*) led to changes in flower color of ornamental gentian plants (Nakatsuka et al. 2008). Naturally, the flower of gentian plants is vivid-blue in color. Accumulation of a polyacrylate delphinidin 'gentiodelphin' in the petals of gentian plants contribute to the flower color. Anthocyanin 5,3'-aromatic acyltransferase (5/3'AT) and flavonoid 3',5'-hydroxylase (F3'5'H) are crucial enzymes for gentiodelphin biosynthesis and their downregulation via RNAi causes modification in flower color (Nakatsuka et al. 2010).

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He et al. (2013) experimented to rebuild the delphinidin pathway, for which they first identified two cultivars of chrysanthemum and isolated seven anthocyanin biosynthesis genes, namely *CmCHS*, *CmF3H*, *CmF3*'H, CmDFR, *CmANS*, *CmCHI* , and *Cm3GT*. Furthermore, the overexpression of the *Senecio cruentus F3′5′H* (*PCFH*) gene and suppression of the *CmF3'H* gene in chrysanthemum resulted in increased cyanidin content with brighter red flower petals but the accumulation of delphinidin has not been reported.

2.4.7 Nutritional improvement

Plants serve as the major source of required nutrients in the human diet. But more than two-thirds of the world's population is deficient in one or more essential mineral elements (White and Broadley 2009). RNAi can be employed to achieve the required levels of nutrients in crops by modifying various biochemical and physiological pathways. Omega-3 fatty acid desaturase (FAD3) enzyme catalyzes the synthesis of α-linolenic acid (18:3) in the polyunsaturated fatty acid synthesis pathway. α-linolenic acid is responsible for instability in soybean (*Glycine* max) and other seed oils. Flores et al. (2008) through siRNA mediated silencing of FAD3 in soybeans significantly decreased the level of α-linolenic acid by 1–3% as compared to other non-transgenic lines. In *Camelina sativa,* the oilseed quality has been improved by downregulating the *fatty acyl-ACPthioesterase* (*FATB*) gene using artificial miRNA (amiFATB). The results showed a considerable decrease in total saturated fatty acids content with a 45% reduction in palmitic acid (16:0) and a 38% reduction in stearic acid (18:0) as compared to wild-type seeds (Ozseyhan et al. 2018).

Kusaba et al. (2003) generated a rice cultivar with low glutenin content (named as LGC-1) through the silencing of the *gluB* gene using hairpin RNA. A high amylose content wheat cultivar has been produced by suppressing the expression of two starch branching enzyme (SBE) II (namely SBEIIa and SBEIIb) in wheat endosperm using RNAi (Regina et al. 2006). In plants starch phosphorylation and starch dephosphorylation act as crucial components in the starch degradation process. Downregulation of *glucan water dikinase* (*GWD*) and *phosphoglucan phosphatase* (*SEX4*) through RNAi resulted in the accumulation of starch in leaves of *Arabidopsis thaliana* and maize (Weise et al. 2012). Carotenoid content in *Brassica napus* was

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elevated by RNAi mediated silencing *of ε-Cyclases* (ε-CYC). Seed obtained by RNAi transgenic *Brassica* lines were found to be rich in β-carotene, zeaxanthin, lutein, and violaxanthin (Yu et al. 2008). Similarly, carotenoid and flavonoid content in tomatoes was increased by knocking down the endogenous photomorphogenesis regulatory gene *DET1* using RNAi (Davuluri et al. 2005).

The phenomenon of accumulation of sucrose and other reducing sugars in potato tubers during storage at low temperatures called 'cold sweetening'. In potato tubers, sugar phosphatase (SPP) plays a significant role in carbohydrate metabolism at room temperature. Downregulation of the SPP gene through RNAi leads to inhibition of cold-induced hexogenesis in transgenic tubers (Chen et al. 2008). RNAi can also be employed for the accumulation of minerals in crops. Aggarwal et al. (2018) through RNAi-mediated downregulation of *inositol pentakisphosphate kinase* (*IPK1*) produced wheat grains having high Zn and Fe content with a reduced level of antinutrient phytic acid (PA).

2.4.8 Secondary metabolite production

Plant secondary metabolites are used in fragrances, drugs, food additives, pigments, and pesticides. Biosynthesis of secondary metabolites is regulated by an array of multiple genes, but sometimes it may get obstructed by certain undesirable compounds. RNAi can be used as an effective approach to suppress the expression of those compounds as well as for secondary metabolite manipulation (Borgio 2009). Allen et al. (2004) reported the replacement of morphine with non-narcotic alkaloid (S)-reticuline in opium poppy (*Papaver somniferum*) through RNAi-mediated silencing of multiple genes participating at different steps in a complex biochemical pathway. They constructed hpRNA for suppressing the expression of all the members of the codeine reductase (COR) gene family. This led to the development of transgenic lines consisting of (S)-reticuline, a non-narcotic alkaloid precursor by replacing morphine, codeine, and opium.

Caffeine acts as a natural stimulant for the central nervous system, respiratory system, and circulatory system. It also lessens the risk of liver cancer, mouth, and throat cancer. Besides this, its excess intake may cause some health issues such as insomnia, nervousness, an upset stomach, restlessness, and muscle tremors. In coffee plants, three enzymes participate in the caffeine biosynthesis viz. CaXMT1, CaMXMT1 (theobromine synthase) and CaDXMT1 (caffeine

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synthase). The RNAi mediated silencing of *CaMXMT1*gene resulted in, 70% decrease in caffeine content, indicating RNAi technology can be employed for the production of decaffeinated coffee beans (Ogita et al. 2003). Similarly, low caffeine content containing tea (*Camellia sinensis*) transgenic lines were developed by downregulating the caffeine synthase (CS) gene using RNAi (Mohanpuria and Kumar 2011).

Salvia miltiorrhiza is a famous Chinese herb, also used in other Asian countries. The production of phenolic acid was enhanced by downregulating the initial enzyme in flavonoid biosynthesis; i.e., *Chalcone synthase* (*CHS*) gene with elicitor treatment of salicylic acid. Results showed a considerable decrease in flavonoid production with an increase in phenolic acid content (Zhang et al. 2015). In several aromatic plants such as spearmint (*Mentha spicata*), tiny specialized structures present for the secondary metabolite production called as peltate glandular trichomes (PGT). Wang et al. (2016) examined the role of transcription factors in the secondary metabolite biosynthesis pathway, for which they isolated and functionally characterized a *MsYABBY5* gene expressed in PGT. Production of terpenes was increased after the suppression of the *MsYABBY5* gene suggesting encoded transcription factors act as a negative regulator for secondary metabolite production.

In papaya (*Carica papaya L.*) plants, *DE-ETIOLATED-1* (*DET1*) gene, which is a negative regulator of photomorphogenesis, was suppressed through RNAi in embryonic callus to study its effects on expression of a gene involved in the biosynthesis pathway of secondary metabolites, results suggested a relationship between photo-regulated pathway and secondary metabolite synthesis (Jamaluddin et al. 2019).

3. Clustered regularly interspaced short palindromic repeat (CRISPR)/CRISPR-associated protein (CRISPR/Cas)

Until 2013, the zinc-finger nucleases (ZFNs) and transcription activator-like effector nucleases (TALENs) were used as the most prevalent gene-editing tools (Kim et al.1996; Christian et al. 2010). These methods of gene editing rely on the use of specific DNA recognition and binding properties of specialized proteins viz. customized homing nuclease (meganuclease), zinc-finger

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nucleases (ZFNs) and transcription activator-like effector nucleases (TALENs). These nucleases are fusion products of domain derived from zinc finger transcription factors or transcription activator-like effectors formulated to identify almost any DNA sequence and the endonuclease domain of class II restriction enzyme which can introduce double-stranded breaks (DSBs) (Puchta and Fauser 2013). CRISPR (Clustered Regularly Interspaced Short Palindromic Repeats) and CRISPR-associated protein-9 (Cas9) nuclease system came out as a viable tool for the targeted gene editing in plants. In the last decade, CRISPR/CAS system has made great achievements in many fields owing to its targeting, efficiency, versatility, and simplicity (Fig. 3).

CRISPR comprises of DNA fragment with short palindromic repeats that are interspaced by the short sequences of variable length regarded as 'non-repetitive' elements or spacers. CRISPR assembly was first recognized in the genome of *Escherichia coli* in 1987 (Ishino et al. 1987). The functional relationship between CRISPR locus and adjacently located CRISPR associated (Cas) genes was identified later (Jansen et al. 2002). The biological function of the CRISPR/Cas system was, however, unknown until 2015. A quantum leap came in the gene-editing technology with the findings that variable spacer sequences are derived from the foreign genome of virus and plasmid, suggesting the role of CRISPR/Cas system as a part of adaptive immunity in prokaryotes (Bolotin et al. 2005; Mojica et al. 2005; Pourcel et al. 2005). The immunity is acquired by the acquisition of short DNA segments of invading viruses and plasmids in between the adjacent repeats as spacers. The CRISPR/Cas system provides immunity by utilizing the RNA-guided nucleases to cleave the genome of invaders in a sequence-specific manner. This was experimentally confirmed in 2007; i.e., phage-resistant bacteria have integrated spacers similar to the nucleic acid sequence of bacteriophage and the phage-resistant phenotype can be altered by insertion or deletion of particular spacers. This implies that CRISPR in association with Cas genes could participate in providing immunity against viruses and plasmids (Barrangou et al. 2007). The motifs associated with spacer precursor (proto-spacers) from the genome of invading viruses were identified at the time of the spacer uptake mechanism. These short stretches of di- or trinucleotides usually have sequence 5'-NGG- 3' and exceptionally 5'-NAG-3'present at or one position downstream to proto-spacers were named as proto-spacers adjacent motifs (PAMs). These motifs (PAMs) play a key role in the identification of proto-spacers as well as assure the correct integration of spacers in between repeated arrays of CRISPR (Bolotin

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et al. 2005; Kunin et al. 2007; Mojica et al. 2009). Specificity is provided by 'Seed Sequence' present approximately 12bp upstream of PAM, which must be complementary to the RNA. Brouns et al. (2008) revealed that a long transcript CRISPR RNA precursor (pre-crRNA) is produced by transcription of CRISPR locus which further processed into a mature crRNA molecule, which serves as single guide RNA (sgRNA). Each crRNA molecule consists of spacers which are flanked by short DNA repeats, this crRNA combines with transactivating CRISPR RNA (tracr RNA), which stimulate Cas9 and mediates the antiviral response. In 2010, it was experimentally proved that CRISPR1/Cas system of *Streptococcus thermophiles* naturally uptake spacers from a self-replicating plasmid containing antibiotic-resistant genes, provided to select transformed bacteria. They also examined *in vivo* that CRISPR1/Cas creates double-stranded breaks at specific sites within proto-spacers, suggesting the molecular basis of CRISPR/Cas system-mediated adaptive immunity (Garneau et al. 2010). As compared to ZFNs and TALENs, the construction of the CRISPR/Cas system is easier as it consists of just a Cas9 protein and a synthetic single-guide RNA (sgRNA), which needs to be designed complementary to the target DNA sequence.

3.1 Mechanism of action

Makarova et al. (2011)**,** classified the CRISPR/Cas system into three distinct polythetic classes named as Type I, II, and III. Cas1 and Cas2 serve as a vital constituent of all three systems as they play a crucial role in the integration of spacers in between the repeated array of CRISPR. Each system consists of its signature proteins and depends on these proteins to generate an immune response against the invading virus or plasmid. In summary,

- ◊ Type I systems contain signature protein Cas3 that consists of both helicase and DNase domain for the degradation of target (Sinkunas et al. 2011). Recently, six subtypes of type I system (Subtype I-A to I-F) have been identified containing a variable number of Cas proteins. Aside from Cas proteins, type I system also encodes for CRISPR associated complex for antiviral defense (Cascade) complex, Cas3 is also the part of this complex.
- ◊ Type II encodes three signature proteins, viz. Cas1, Cas2 and Cas9, and sometimes a fourth protein; i.e., Csn2 and Cas4. Cas9 is a multifunctional protein that in Type II system plays a crucial role in adaptation to the degradation of the target along with trans-encoded small RNA (tracr RNA)(Jinek et al. 2012; Deltcheva et al. 2011; Garneau et al.

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2010; Wei et al. 2015). Three subtypes of type II system have been discovered namely type II-A, type II-B, and type II-C (Chylinski et al. 2013; Koonin and Makarova 2013).

◊ Type III is defined by the presence of Cas10, whose function is still unclear. Two subtypes of type III system(type III-A and type III-B) have identified (Rath et al. 2015).

Type I and II system targets DNA degradation but exceptionally, the type III system targets DNA as well as RNA. The most widely used system is the type II CRISPR/Cas9 system from *Streptococcus pyogenes* (Jinek et al. 2012). Until now, type II system has been studied in bacteria but type I and type III systems have marked their presence in both bacteria and archaea (Makarova et al. 2011). The general mechanism of action of the CRISPR/Cas system involves three stages; i.e., adaption, expression, and interference. The proteins involved in the adaption stage (namely, Cas1 and Cas2) are highly conserved whereas in expression and interference stage the proteins vary greatly between the organisms. Each stage details are given below:

1. Adaption stage: The short pieces of DNA homologous to the genomic sequence of invading virus or plasmid get incorporated at the leader side of CRISPR locus. A new spacer unit is created by duplication of repeats at every integration step. In type, I and III CRISPR/Cas system, selection of proto-spacers occur by the recognition of PAMs present on or near the location of proto-spacers of invading genetic element (Mojica et al. 2009; Marraffini and Sontheimer 2008; Marraffini and Sontheimer 2010). After the recognition, Cas1 and Cas2 proteins help in the integration of proto-spacers in between the repeats array of CRISPR.
2. Expression stage: At this stage, the expression of spacer takes place via transcription of CRISPR locus and leads to the generation of a long-transcript of pre-CRISPR RNA (pre-crRNA), which is processed into short crRNA by endoribonucleases. In the type I CRISPR/Cas system, pre-crRNA binds with CRISPR associated complex for antiviral defense (Cascade) complex, processed into crRNA by cleavage through Cas6e and Cas6f. The crRNA produced has an 8-nt repeat fragment at 5'end and the fragment left forms the hairpin structure on 3' end. In type II CRISPR/Cas system, a repeated fragment of pre-crRNA pairs with the trans-encoded small RNA (tracer RNA), which further cleaved by RNase III in the presence of Cas9 (Deltcheva et al. 2011). Consequent, cleavage at a

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fixed distance in spacers may lead to the maturation. Type III system uses Cas6 protein for processing to crRNA, but afterward, crRNA transferred to a different complex of Cas proteins namely Csm in subtype III-A systems and Cmr in Subtype III-B. Further, cleavage occurs at 3' end in subtype III-B subsystems (Hale et al. 2009).

3. Interference stage: After the expression, invading DNA or RNA is targeted and cleaved within proto-spacer sequences. The crRNA acts as a single guide RNA and guides the Cas protein towards the complementary target sequences of invading genome of virus or plasmid. In type I systems, Cascade complex guided by crRNA towards complementary target DNA, and invading DNA possibly cleaved by Cas3 protein. Cas9 protein loaded with crRNA cleaves the target DNA in type II systems. The subtype of type III system, III-A systems target DNA (Marraffini and Sontheimer 2008) whereas III-B systems target RNA (Hale et al. 2009).

3.2 Applications

Progression of CRISPR/Cas system from biological defense phenomenon to gene-editing tool came into light when it was revealed that genome sequence can be remodeled by simply modifying the 20nt in the crRNA, and fusing it with tracr RNA to make a single chimeric guide RNA (gRNA). This led to the reduction of the three-component system to a two-component CRISPR/Cas system (Jinek et al. 2012). Unlike Cas3 protein, which degrades the target completely, Cas9 introduces single double-stranded breaks (DSBs) in DNA which is a salient feature to be an efficient gene-editing tool. DSBs induced in DNA triggers the DNA repair pathways in the cell, and CRISPR/Cas9 manipulates these pathways to alter the genome. Two main pathways involved in the DNA repair viz. Non-homologous end joining (NHEJ) and homology-directed repair (HDR). NHEJ is more error-prone as while the cuts, insertion, or deletion (InDels) mutation may take place, resulting in gene knock-out of mutation occurs in the coding region or the production of a cripple gene product. HDR utilizes another piece of DNA homologous to target DNA to repair DSBs. As in HDR, incorporation of DNA element is taking place through recombination, so any kind of insertion, deletion, or alteration in sequence can be done (Cong et al. 2013; Mali et al. 2013). Off-target cleavage can be avoided by selecting unique target sites adjacent to PAM (Jinek et al. 2012). These approaches have previously been studied using ZFNs and TALENs but

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comparatively, Cas9 is simple to construct and use as well as it can target multiple genes simultaneously (Cong et al. 2013).

Owing to high efficiency, versatility, and simplicity of the CRISPR/Cas system can be employed for developing new cultivars (Table 2). It has been widely used in many plants such as *Arabidopsis thaliana, Nicotiana benthamiana, Oryza sativa* (Song et al. 2016), and *Solanum tuberosum*.

3.2.1 Yield improvement

In several plants, seasonal change in day-length may trigger flowering and day-length sensitivity, limiting their geographical range of cultivation. CRISPR/Cas9-mediated mutation in *SPG5*, which is a repressor of florigen paralog and flowering resulted in rapid flowering with enhancement in compact determinate growth habit of field tomato (Soyk et al. 2016). Li et al. (2017a), using the RNA-guided Cas9 system, demonstrated that this technology can be used *in vivo* as the desired target mutator (DTM) to develop mutated maize germplasm. For hybrid breeding in crops, photoperiod and thermosensitive genetic male sterility (PGMS and TGMS) are the two main components. To improve the yield potential of rice, the development of hybrid rice is important. Hybrid rice breeding relies on the two-line system, and the generation of thermo-sensitive genetic male sterile is widely used male sterile in this system. TMS5 broadly applied the *TGMS* gene from China was manipulated using the CRISPR/Cas system to develop new 'transgene clean' TGMS lines. 11 novel cultivars of TGMS were developed in one year, indicating the ability of the CRISPR/Cas system for improving efficiency in hybrid rice breeding (Zhou et al. 2016). Li et al. (2016), employed CRISPR/Cas gene-editing tool to mutate *Gna1*, *DEP1*, *GS3* and *IPA1* genes of rice cultivar Zhonghua 11, resulted in T2 generation of *gna1*, *dep1* and *gs3* mutants showing characteristics like enhanced grain number, dense erect panicles and larger grain size, respectively.

3.2.2 Abiotic stress tolerance

CRISPR/Cas9 system has enormous potential to generate crops tolerant to abiotic stresses. CRISPR/Cas9-mediated knock-out of rice annexin gene *OsAnn3*, led to the development of

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mutant lines tolerant to cold. Thus, indicating the involvement of *OsAnn3* in cold tolerance of rice (Shen et al. 2017). In *Arabidopsis thaliana*, C-repeat binding factors (CBFs) plays a decisive role in cold-stress tolerance. However, the precise function of these factors is unclear owing to the lack of null cbf triplet mutants. Thus, CRISPR/Cas9 has been employed to produce cbf 1,3 double and cbf 1,2,3 triple mutants by disrupting *CBF1* or *CBF1*/ *CBF2* in a *cbf3* T-DNA insertion mutant (Jia et al. 2016).

Mitogen-activated protein kinases (MAPK1) signaling molecules play a significant role in drought stress tolerance. In tomato, drought stress causes the accumulation of reactive oxygen species (ROS), which causes oxidative damage in tomatoes. *SlMAPK3* mutants generated by CRISPR/Cas gene editing led to more tolerance in the tomato plants (Wang et al. 2017). Maize *ARGOS8* acts as a negative regulator of ethylene response. New variants of *ARGOS8* were developed with native maize GOS2 promoter through CRISPR/Cas advanced breeding technology for the production of drought-tolerant crops. The field study showed that the *ARGOS8* variant of maize has increased grain yield significantly as compared to the wild type under flowering stress-condition (Shi et al. 2016).

SNF-1 related protein kinases 2 (SnRK2) serve as the main regulator of hyper-osmotic stress signaling and ABA-dependent development in plants. SnRK2 and osmotic stress/ABA activated protein kinase 2 (SAPK2) can be the primary mediator of ABA signaling in rice subclass- I and II. Lou et al. (2017), examined the functional role of *SAPK2* by producing loss-of-function mutants using CRISPR/Cas technology. When drought, high-salinity, and polyethylene glycol (PEG) stresses were given, *SAPK2* expression was highly up-regulated. The *SAPK2* mutants showed ABA-insensitive phenotype during germination and post-germination stage, suggesting the importance of ABA-mediated seed dormancy. Moreover, it has been observed that *SAPK2* increases the tolerance of rice plants to salt and PEG stress.

3.2.3 Biotic stress tolerance

CRISPR/Cas9 system has originally emerged as a part of adaptive immunity in bacteria and archaea. Over the past years, it has been explored for targeted gene editing in various plants to provide resistance against biotic stresses. CRISPR/Cas9 system used to confer resistance

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against *Tomato yellow leaf curl virus* (TYLCV) into *Nicotiana benthamiana* plants by designing sgRNA consisting of coding and non-coding sequences of TYLCV, resulted in reduced viral DNA accumulation with considerable attenuation in symptoms of infection (Ali et al. 2015). Similarly, using the sgRNA-Cas9 system in *Nicotiana benthamiana beet severe curly top virus* (BSCTV) accumulation has also been reduced (Ji et al. 2015). Virus-resistant cucumber (*Cucumis sativus* L.) cultivars were developed using the sgRNA-Cas9 system to disrupt the function of the recessive eIF4E gene. Resultant non-transgenic homozygous T3 progenies showed resistance against *Cucumber vein yellowing virus* (Ipomovirus) infection, potyviruses such as *Zucchini yellow mosaic virus* and *Papaya ringspot mosaic virus-W* (Chandrasekaran et al. 2016). Likewise, Pyott et al. (2016) employed CRISPR/Cas9 technology to introduce deleterious site-specific mutation in *eIF(iso)4E* locus of *Arabidopsis thaliana* to develop transgenic lines completely resistant against *Turnip mosaic virus* (TuMV), which is a major pathogen for vegetables. These findings suggest that CRISPR/Cas9 system is an innovative approach to generate potyvirus resistant agronomically important crops without incorporating transgenes. Zhan et al. (2019) used CRISPR/Cas 13a in potato plants to develop resistance against *Potato virus Y* (PVY). Reduced accumulation of virus and symptoms observed in transgenic potato lines.

In *Arabidopsis thaliana, enhanced disease resistance 1* (*EDR1*) gene acts as a negative regulator of defense response against powdery mildew. CRISPR/Cas9 technology was employed to develop the *Taedr1* wheat plants by altering the three homoeologs of wheat *EDR1*. No off-target mutations have been detected in *Taedr1* mutants and were found to be resistant to powdery mildew (Zhang et al. 2017).

4. Conclusion and future prospects

In the 21st century, the foremost task for the agriculture industry is to provide food security to the rapidly expanding population globally. Besides, developing countries are also facing malnutrition. Hence, to ensure an adequate supply of balanced food to the world, there is an urgent need to develop biofortified staple food, vegetables, and fruits, enriched in all the essential compounds and mineral elements. The development of cultivars resistant to biotic stresses and tolerant to abiotic stresses such as changing environmental conditions, high

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temperature, drought, flood, oxidative stresses, high salt concentration, and heavy metal-polluted soil can be a setback for world food security, malnutrition, and famine problems. Feasibility of using RNAi and CRISPR/Cas9 technology has become a topic of current interest in the last few years (Fig. 4). These approaches hold great potential to develop crops with high-value agronomic traits by targeting their broad range of targets, accelerating crop improvement schemes, and increasing their effectiveness. These technologies may help in supporting food security in both developed as well as developing countries. Studies concern to gene silencing and gene deletion or disruption have become essential to analyze the gene function in crops that further help to design better gene-editing strategies. Both RNAi and CRISPR/Cas will bring a gene revolution in breeding crops with desired traits including quality.

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Authors' contributions

MR, KC and MK wrote the manuscript and draw the illustrations. AC, RO, VV assisted in manuscript writing and editing. NP and AC conceptualized, supervised and edited the manuscript. All authors read and approved the final manuscript.

Conflict of interest

The authors declare that they have no conflict of interest.

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Table 1. Crops with improved stress tolerance through RNAi

Trait(s)	Crop improved	Resistance against	Targeted gene(s)	References
Virus resistance	*Nicotiana bethamiana*	Chilli-infecting begomoviruses	*AC1* *AC2* *βC1*	(Kumar at al. 2015)
	Triticum spp.	*Triticum mosaic virus* (TMV)	*Coat protein* (*CP*)	(Shoup Rupp et al. 2016)
	Oryza sativa	*Rice black streak dwarf virus* (RBSDV)	*S7-2* *S8*	(Ahmed et al. 2017)
	Solanum tuberosum	*Potato virus X* (PVX), *Potato virus Y* (PVY) *Potato virus S* (PVS)	*CP*	(Hameed et al. 2017)
	Glycine max	*Soybean mosaic virus* (SMV)	*SMV P3* cistron	(Yang et al. 2018)
		Mungbean yellow mosaic virus (MYMIV)	*CP*	(Kumari et al. 2018)
	Arachis hypogaea	*Tobacco streak virus* (TSV)	*CP*	(Senthilraja et al. 2018)
	Oryza sativa	*Rice tungroo bacilliform virus* (RTBV) *Rice tungroo spherical virus* (RTSV)	*Coat protein 3* *CP3*	(Malathi et al. 2019)
	Glycine max	*Soybean mosaic virus* (SMV)	*eIF4E1*	(Gao et al. 2020)
	Nicotiana bethamiana	*Tomato yellow leaf curl Thailand virus* (TYLCTV)	*GSA*	(Tzean et al. 2020)
Bacterial resistance	*Arabidopsis thaliana*	*Agrobacterium tumefaciens*	*iaaM* *ipt*	(Escobar et al. 2001)
		Pseudomonas syringae	*PPRL*	(Katiyar-agarwal et al. 2006)
	Citrus limon	*Xanthomonas citri*	*CalS1*	(Enrique et al. 2011)
Fungal resistance	*Solanum tuberosum*	*Phytophthora infestans*	*Avr3a*	(Sanju et al. 2015)
	Triticum aestivum	*Fusarium graminearum*	*Chs 3b*	(Cheng et al. 2015)

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	Musa spp.	*Fusarium oxysporum* f. sp. *cubense* (Foc)	*Foc velvet protein*	(Ghag et al. 2015)
	Nictotiana tabacum	*Sclerotinia sclerotiorum*	*Chs*	(Andrade et al. 2016)
	Solanum lycopersicum	*Fusarium oxysporum*	*Fow2* *chs V*	(Bharti et al. 2017)
	Oryza sativa	*Magnaporthe oryzae*	*MoABC1* *MoMAC1* *MoPMK1*	(Zhu et al. 2017)
		Rhizoctonia solani	*RPMK1-1* *RPMK1-2*	(Tiwari et al. 2017)
	Zea mays	*Aspergillus flavus*	*ZmPRms*	(Majumdar et al. 2017)
	Solanum lycopersicum	*Fusarium oxysporum*	*Fmk1* *Hog1* *Pbs2*	(Pareek and Rajam 2017)
	Zea mays	*Aspergillus flavus*	*Amy1*	(Gilbert et al. 2018)
	Solanum tuberosum	*Phytophthora infestans* *Alternaria solani*	*PVS1* *PVS2* *PVS3* *PVS4*	(Yoshioka et al. 2019)
	Glycine max	*Phytophthora sojae*	*GmSnRK1.1*	(Wang et al. 2019a)
	Solanum lycopersicum	*Fusarium oxysporum*	*ODC*	(Singh et al. 2020)
Insect resistance	*Solanum lycopersicum*	*Helicoverpa armigera*	*HaCHI*	(Mamta et al. 2016)
	Nictotiana tabacum	*Bemisia tabaci*	*AChE* *EcR*	(Malik et al. 2016)
	Lettuce	*Bemisia tabaci*	*V-ATPase*	(Ibrahim et al. 2017)
	Arabidopsis thaliana	*Myzus persicae*	*MyCP*	(Bhatia and Bhattacharya 2018)
	Brassica rapa	*Tetranychus urticae*	*COPB2*	(Shin et al. 2020)
Nematodes Resistance	*Solanum lycopersicum*	*Meloidogyne incognita*	*Mi-cpl1*	(Dutta et al. 2015)

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	Nicotiana benthamiana	*Radopholus similis*	*Rs-cps*	(Li et al. 2017b)
	Solanum lycopersicum	*Meloidogyne incognita*	*PolA1*	(Chukwurah et al. 2019)
	Glycine max	*Heterodera glycines*	*Hg16B09*	(Hu et al. 2019)
			HgY25 HgPrp17	(Tian et al. 2019)
	Arabidopsis thaliana	*Meloidogyne incognita*	*Mi-msp3 Mi-msp 5 Mi-msp18 Mi-msp24*	(Joshi et al. 2020)
Abiotic stress tolerance	*Nictotiana tabacum*	Salt tolerance	*Nτε-LCY*	(Shi et al. 2015)
	Oryza sativa	Salt tolerance	*OsPEX11*	(Cui et al. 2016)
	Brassica rapa	Salt tolerance	*GIGANTEA* (*GI*)	(Kim et al. 2016)
	Arabidopsis thaliana	Drought tolerance	*PAD4 LSD1 EDS1*	(Szechyn´ska-Hebda et al. 2016)
	Oryza sativa	Drought tolerance	*OsGRXS17*	(Hu et al. 2017)
	Oryza sativa	Drought tolerance	*OsDSR-1*	(Yin et al. 2017)
	Oryza sativa	Drought tolerance	*OsERF101*	(Jin et al. 2018)
	Solanum lycopersicum	Drought and salt tolerance	*SlbZIP1*	(Zhu et al. 2018)
	Nictotiana tabacum	Drought tolerance	*BrDST71*	(Park et al. 2018)
	Triticum aestivum	Salt tolerance	*TaPUB-1*	(Wang et al. 2019b)
	Arabidopsis thaliana	Osmotic tolerance	*WZY2*	(Yu et al. 2019)

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Table 2: CRISPR/Cas9 system-mediated gene editing in crops

Trait(s)	Crop used	Targeted gene(s)	References
Drought tolerance	*Zea mays* (Maize)	*ARGOS8*	(Shi et al. 2016)
Turnip mosaic virus (TMV) resistance	*Arabidopsis thaliana*	*eIF(iso)4E*	(Pyott et al. 2016)
Cucumber vein yellowing virus (CMYV) resistance	*Cucumis sativus*	*eIF4E*	(Chandrasekaran et al. 2016)
Drought tolerance	*Solanum lycopersicum*	*SlMAPK3*	(Wang et al. 2017)
Cold tolerance	*Oryza sativa*	*OsAnn3*	(Shen et al. 2017)
Parthenocarpic fruit development	*Solanum lycopersicum*	*SlIAA9*	(Ueta et al. 2017)
Chilling stress tolerance	*Solanum lycopersicum*	*SlCBF1*	(Li et al. 2018)
Tomato yellow leaf curl virus (TYLCV) resistance	*Solanum lycopersicum* *Nicotiana benthamiana*	*Coat protein* (*CP*) *Replicase* (*Rep*)	(Tashkandi et al. 2018)
Cauliflower mosaic virus (CMV) resistance	*Arabidopsis thaliana*	*CaMV CP*	(Liu et al. 2018)
Rice tungro spherical virus (RTSV) resistance	*Oryza sativa*	*eIF4G*	(Macovei et al. 2018)
Salt tolerance		*OsRR22*	(Zhang et al. 2019)
Male-sterile development	*Triticum aestivum*	*Ms1*	(Okada et al. 2019)
Heat stress tolerance	*Solanum lycopersicum*	*SlMAPK3*	(Yu et al. 2019)
Drought and salt stress		*DAP4*	(Chen et al.

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tolerance	*Arabidopsis thaliana*	*SOD7*	2019)
Drought tolerance		*AREB1*	(Felipe et al. 2019)
Wheat dwarf virus (WDV) resistance	*Hordeum vulgare*	*CP* *Rep*/*Rep4*	(Kis et al. 2019)
Yield improvement	*Brassica napus*	*BnaMAX1*	(Zheng et al. 2020
Yield improvement Stress tolerance	*Oryza sativa* (Nippobare)	*OsPIN5b* *GS3* *OsMYB30*	(Zeng et al. 2020)
Yield improvement	*Oryza sativa*	*Cyt P450 homeologs* *OsBADH2*	(Usman et al. 2020)
Drought and stress tolerance		*OsDST*	(Kumar et al. 2020)
Tomato yellow leaf curl virus (TYLCV) resistance	*Solanum lycopersicum*	*rgsCaM*	(Ghorbani Faal et al. 2020)
Soyabean mosaic virus (SMV) resistance	*Glycine max*	*GmF3H1* *GmF3H2* *GmFNSII-1*	(Zhang et al. 2020)

Figure legends:

Fig. 1: Mechanism of miRNA biogenesis and gene silencing

Fig. 2: siRNA biogenesis and gene silencing

Fig 3: Applications of CRISPR/Cas9 system in crop improvement

Fig 4: RNA interference Vs CRISPR/ Cas9 system

Table legends:

Table 1. Crops with improved stress tolerance through RNAi

Table 2: CRISPR/Cas9 system-mediated gene editing in crops

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