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CRISPR/dCas9-based systems: Mechanisms and applications in plant sciences

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Abstract
RNA-guided genomic transcriptional regulation tools, namely Clustered Regularly Interspaced Short Palindromic Repeats interference (CRISPRi) and CRISPR-mediated gene activation (CRISPRa), are a powerful technology for the field of functional genomics. Deriving from the CRISPR/Cas9 system, both systems comprise a catalytically dead Cas9 (dCas9) and a single guide RNA (sgRNA). This type of dCas9 is incapable of cleaving DNA but retains its ability to specifically bind to DNA. The binding of the dCas9/sgRNA complex to a target gene results in transcriptional interference. The CRISPR/dCas9 system has been explored as a tool for transcriptional modulation and genome imaging. Despite its potential applications and benefits, the challenges and limitations faced by the CRISPR/dCas9 system include the off-target effects, PAM sequence requirement, efficient delivery methods, and the CRISPR/dCas9-interfered crops being labeled as genetically modified organisms in several countries. This review highlights the progression of CRISPR/dCas9 technology as well as its applications and potential challenges in crop improvement.

Keywords: CRISPR interference; CRISPR/dCas9 system; crop improvement; gene silencing; RNAi; transcriptional regulation

Introduction

Recent advancements in omics-based technologies and bioinformatics methodologies have facilitated the ever-growing field of functional genomics and system biology (Guigo & De Hoon, 2018; Yadav & Tripathi, 2018). With the help of such advanced technologies and gene-regulating tools, such as RNA interference (RNAi), zinc finger nucleases (ZFNs), and transcription activator-like effector nucleases (TALENs), researchers are now able to investigate the roles of specific genes in a cell or an organism. RNAi is a powerful method for gene function validation. This technology was first discovered in *Caenorhabditis elegans* (Fire et al., 1998; Lee et al., 1993). RNAi is a conserved eukaryotic mechanism that uses small RNA molecules, such as small interfering RNAs (siRNAs) and microRNAs (miRNAs), to inhibit the translation of the target proteins. This method has been successfully applied in several crops to improve their resistance against pathogens like fungi, bacteria, and insects (Singh et al., 2020) as well as abiotic stress tolerance, such as salinity and drought (Hanly et al., 2020). Despite its popularity, RNAi has several limitations, including inconsistency and

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incompleteness of knockdowns, potential non-specificity or off-targeting effect, and inefficient delivery methods (Mamta & Rajam, 2017). Genome editing tools, such as ZFNs and TALENs, seem a better alternative to RNAi, since these techniques induce fewer off-target effects. ZFNs and TALENs comprise a FokI nuclease domain and a DNA-binding domain. As the FokI nuclease domain requires dimerization to become active, a tail-to-tail orientation with appropriate spacing needs to be designed to enable dimerization of the FokI domains. This requirement provides specificity to ZFNs and TALENs. However, the synthesis of active nucleases is relatively expensive and difficult (Wada et al., 2020).

Deriving from the Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR)/Cas9 system, the newly developed CRISPR/dCas9 has been repurposed for transcriptional regulation. This system comprises three major components: a catalytically inactive Cas9 (dCas9) protein, a customizable single guide RNA (sgRNA) that complements the promoter region of a gene, and transcriptional effectors, either transcriptional activators (CRISPR activation; CRISPRa) or repressors (CRISPR interference; CRISPRi). The binding of dCas9/sgRNA and transcriptional effector complex to the promoter region of the downstream target genes results in transcriptional interference by blocking RNA polymerase binding or elongation. The CRISPRi functions analogously to RNAi in which both systems aim to silence or knockdown gene expression but possess different mechanisms and principles (Boettcher & Michael, 2015). In essence, the CRISPRi method suppresses gene expression at a DNA level by preventing transcription, whereas RNAi uses a posttranscriptional mechanism by cleaving transcribed mRNAs.

CRISPR/dCas9 technology has revolutionized the fields of functional genomics, as it provides a simple, efficient, and less expensive targeted activation or repression of gene expression (Xu & Qi, 2019). In this paper, we briefly discuss the discovery and principles of CRISPR as well as the development and recent progress of CRISPR/dCas9 technology. This review also highlights the applications and challenges of CRISPR/dCas9 in plant research.

The CRISPR/Cas system: Discovery and principle

CRISPR was first discovered unintentionally in the genome of *Escherichia coli* by Ishino et al. (1987) while sequencing the *iap* gene. The authors found an unusual series of tandemly repetitive 29 nucleotides (nt) DNA sequences interspaced with 32-nt spacer sequences downstream of the gene (Ishino et al., 1987). They have no clue about the biological function of these repeats since it lacks sequence homology to other known sequences at that time. Two years later, Nakata et al. (1989) observed similar interrupted clustered repeats in other *E. coli* strains as well as in the closely related species of *Salmonella typhimurium* and *Shigella dysenteriae*. In 1993, a group of scientists led by Jan D. A. van Embden detected multiple direct repeats with varying spacer sequences of 35 to 41 bp in different strains of *Mycobacterium tuberculosis* (Groenen et al., 1993; van Soolingen et al., 1993). While sequencing numerous fragments in the genome of *Haloferax mediterranei*, Mojica et al. (1995) discovered long tandem repeats. This marked the first time that direct repeats were detected in archaea. Mojica et al. (2000) classified such interspaced repeat sequence as short regularly spaced repeats (SRSRs). To avoid naming confusion in future studies, Jansen et al. (2002) together with Mojica and colleagues renamed these sequences as clustered regularly interspaced short palindromic repeats (CRISPRs). The CRISPRs-associated genes (Cas), *cas1* to *cas4*, were also identified in the same year by Jansen et al. (2002).

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In 2005, it was discovered that the spacers within CRISPRs were derived from invading phages and plasmids (Bolotin et al., 2005; Mojica et al., 2005; Pourcel et al., 2005). These findings provided a clue that CRISPR/Cas could be an adaptive immunity system in prokaryotes. The role of CRISPR/Cas systems as an adaptive immunity has later been supported by experimental findings from several research groups (Makarova et al., 2006; Barrangou et al., 2007; Deveau et al., 2008; Horvath et al., 2008). These authors found that new spacer sequences from the infecting phage are acquired into bacterial CRISPR array.

CRISPR/Cas-mediated immunity has three stages: adaptation, expression, and interference [reviewed by Ishino et al. (2018)]. The first stage involves spacer acquisition, also known as the adaption process, where phage DNA fragments are inserted into the spacer region in the CRISPR locus and stored in the host chromosome at the proximal end of the CRISPR array. The second stage involves the transcription and maturation of CRISPR RNAs (crRNAs). In the final interference stage, the mature crRNAs guide Cas nucleases to cleave the complementary target protospacers sequence of foreign DNA or plasmid.

The CRISPR/Cas systems can be classified into three major types (Types I, II, and III). Type I and III systems employ multi-Cas proteins for target recognition and cleavage. For example, the Type I system uses Cas3 to cleave target DNA (He et al., 2020), whereas the Type III system utilizes Cas10 with the aid of polymerase and repeat-associated mysterious proteins (RAMPs) to cleave RNA and DNA (Dorsey et al., 2019). In comparison, the Type II system only needs a single effector protein (Cas9) to accomplish the interference and thus, is relatively simple to be engineered to function as a genome-editing tool. In the CRISPR/Cas systems, a trans-activating crRNA (tracrRNA) will bind to the repeat sequence of pre-crRNA to form mature crRNAs with the aid of Cas9 and endoribonuclease III (RNase III) (Deltcheva et al., 2011). The mature dual tracrRNA:crRNAs form a complex with a Cas9 protein that can recognize protospacer adjacent motif (PAM) and cleave specifically at 3 bp before the PAM site of the double-stranded DNA (Gleditzsch et al., 2019). In 2012, Emmanuelle Charpentier and Jennifer A. Doudna published a landmark paper (Jinek et al., 2012), detailing the application of the class II CRISPR/Cas9 system for gene editing. This ground-breaking discovery has earned these two scientists the 2020 Nobel Prize in Chemistry.

The current CRISPR/Cas9 system and strategies to mitigate off-target effects

The newly developed CRISPR/Cas system replaces the dual-tracrRNA:crRNA with an artificial sgRNA which can be easily altered to complement the targeted DNA sequence (the region 20-nt upstream of the PAM site) to induce double-stranded breaks (DSBs) at the desired position (De Pater et al., 2018). The DSBs will then be repaired by nonhomologous end-joining (NHEJ) or homology-directed repair (HDR), resulting in the formation of insertions or deletions in coding regions (Zaboikin et al., 2017). CRISPR/Cas9 is now widely used to study gene function and develop genetically modified (GM) organisms. Its high frequency of off-target activity, however, has become a major concern. Many efforts have been made to improve the specificity of the CRISPR/Cas9 system for gene editing. These include modifying Cas9, designing sgRNA, and utilizing Cas9 orthologs from different variants of bacteria.

The structure of the Cas9 has been modified to reduce the off-target effect. For instance, the D10A Cas9 nickase (nCas9), an example of Cas9 mutants, has been shown to have a lower off-target rate because of the structural changes in their binding region (Shen et al., 2014). Instead of directly inducing DSB, nCas9 only produces a nick or single-stranded break at the target site. The paired binding of the nCas9 on the opposite

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strand produces DSB at a higher specificity with reduced potential off-targets by doubling the recognition site of the target gene (Schiml et al., 2016). Nishimasu et al. (2018) developed an engineered Cas9, *Streptococcus pyogenes* Cas9 (SpCas9)-NG that can recognize NG-PAM instead of NGG-PAM, to expand the targeting range and improve its compatibility to the target genomic loci. This approach has also been used to edit genes in *Arabidopsis* (Ge et al., 2019) and rice (Hua et al., 2019). Other modified Cas9 proteins, such as enhanced-specificity eSpCas9 variant (Slaymaker et al., 2016), hyper-accurate Cas9 variant, HypaCas9 (Chen et al., 2017), and high-fidelity SpCas9-HF1 (Kleinstiver et al., 2016), have also been reported. These modified Cas9 proteins were shown to nearly entirely avoid nonspecific DNA editing. Zhang et al. (2017) demonstrated that the on-target/off-target indel frequency ratio for eSpCas9 and SpCas9-HF1 was 273-fold higher than that for the wild-type SpCas9, showing its high efficiency in gene editing.

Besides modifying the nCas9, a new technique called base editing has also been developed. This approach allows direct conversion of one target DNA base into another base without DSBs (Komor et al., 2016). By fusing nCas9 with adenine base editors, CRISPR-mediated base editing enables the conversion of A-T to G-C, while fusion with cytosine base editors can alter a A-T base pair into a C-C base pair (Marzec & Hensel, 2018; Molla & Yang, 2019). Shimatani et al. (2017) successfully developed herbicide-resistant rice plants using a base-editing approach through a C287T mutation on acetolactate synthase. The C287T mutation leading to an A96V amino acid substitution endows rice plants with a resistance to herbicide imazamox. Despite its great efficiency in editing the DNA, the ability of CRISPR/Cas9 DNA base-editing technology to generate precise base edits beyond the four transition mutations is still a major limitation. Prime editing, another recent DSB-free method, has been developed to overcome these flaws. This method employs an engineered reverse transcriptase fused to nCas9 and a prime editing guide RNA (pegRNA) (Anzalone et al., 2019). The pegRNA differs from sgRNAs as it comprises not only the guide sequence that can recognize the target sites but also a reverse transcriptase template spelling the desired genetic changes. Lin et al. (2020) recently adapted prime editors to induce point mutations, insertions, and deletions in rice. Through this approach, all 12 kinds of base-to-base substitutions, as well as multiple base substitutions, insertions, and deletions, were detected. The authors reported that the frequency of prime editing induced by this prime editor was up to 21.8% (Lin et al., 2020). Similar findings have been reported from other researchers (H. Li et al., 2020; Tang et al., 2020). Although prime editing has several advantages compared with other techniques, including enabling precise sequence deletion, addition, and substitution, this technique is still in its infancy. The specificity and potential for off-target modifications of this technology have yet to be investigated.

sgRNA is an important component in the CRISPR system. As it functions as a guide to Cas9, the design of sgRNA is crucial to reduce the off-target mutation. For instance, sgRNAs with high GC content (40-60%) have been shown to improve the on-target activities in wheat (Wang et al., 2014). If the high percentage of GC is more proximal to the PAM site, the efficiency of on-target gene editing would be higher (Ren et al., 2014). The length of gRNA is another important aspect for the occurrence of unwanted mutations. Ran et al. (2013) found that a shorter length of sgRNA (17 or 18 bp instead of 20 bp) exhibited a 500-fold decrease in off-target events while maintaining on-target accuracy. A recent strategy utilizing the dead truncated sgRNA [dead RNA off-target suppression (dOTS)] has been shown to reduce off-target effects and increase on-target activity by 40-fold (Rose et al., 2020). sgRNAs can also be modified

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chemically by incorporating substances, such as 2'-O-methyl-3'-phosphonoacetate, into the sgRNA ribose-phosphate backbone to mitigate the off-target effects (Ryan et al., 2018). Through this modification, the off-target cleavage was significantly reduced up to 120-fold while maintaining its on-target performance (Ryan et al., 2018). Other modifications include the partial substitution of crRNAs with DNA (Yin et al., 2018), thiophosphonoacetate linkages at the termini (Hendel et al., 2015) or internal residues (Yin et al., 2017), site-specific incorporation of 2'-4' bridged nucleic acids (Cromwell et al., 2018), as well as 2′-O-methyl, 2′-4′ bridged nucleic acid and phosphorothioate linkages (Rahdar et al., 2015).

The requirement of PAM sequence of 5′-NGG-3′ has restrained the Cas9 targeting range. Given that different bacterial strains contain Cas9 proteins recognizing different target PAM sequences, the use of Cas9 orthologs from other bacteria can overcome this limitation. The Cas9 proteins obtained from *Staphylococcus aureus* (SaCas9) and *Neisseria meningitidis* (NmeCas9) were found to recognize the PAM sequence of 5'-NNGRRT (Ran et al., 2015) and 5'-NNNNGATT (Lee et al., 2016), respectively. The introduction of Cas9 orthologs in an organism may not interfere with Cas9. As shown by Steinert et al. (2015), SaCas9 and Cas9 did not interfere with each other, indicating the possibility of editing target regions using different Cas9 orthologs. The use of the NmeCas9 ortholog has significantly reduced the off-target cleavage and increased the target specificity in mammalian cells by exhibiting lower tolerance to base mismatches and DNA bulges (Lee et al., 2016). Müller et al. (2016) edited the *PRKDC* and *CARD11* genes using Cas9 cassettes isolated from *Streptococcus thermophilus* (St1Cas9 and St3Cas9), where the authors found that only a few or no off-target effects have been detected. The recent discovery of the smallest Cas9 ortholog, *Campylobacter jejuni* CAS9 (CjCas9), has also greatly improved the off-target effect without compromising its on-target activity (Dugar et al., 2018).

Inactive CRISPR-associated nucleases: A transcriptional regulator

Besides being a molecular scissor, CRISPR technology has been developed into a sequence-specific and non-mutagenic gene regulation tool to regulate both transcriptional and epigenetic processes. The use of CRISPR/dCas9 technology was first reported by Qi et al. in 2013 (Qi et al., 2013). This technology exploits the deactivated variants of the Cas9 enzyme (dCas9), guided by a sgRNA to form a dCas9/sgRNA complex, that is incapable of cleaving DNA but retains its ability to specifically bind to DNA. By pairing with a transcriptional effector, either repressor or activator, this complex can specifically bind to the target gene at the promoter or coding sequence to interfere the binding of RNA polymerase (RNAP) (Figure 1). Without the binding of RNA polymerase and transcription factors, the transcription of the target gene is inhibited (Khatodia et al., 2016). dCas9 can also be fused to an epigenetic modulator, such as methylation enzyme, to generate dCas9-tethered epigenetic enzymes for targeted regulation at defined genomic loci (O'Geen et al., 2017). The CRISPR/dCas9 system contains three main components: (i) sgRNA, (ii) dCas9, and (iii) the transcriptional effector, which will be discussed below.

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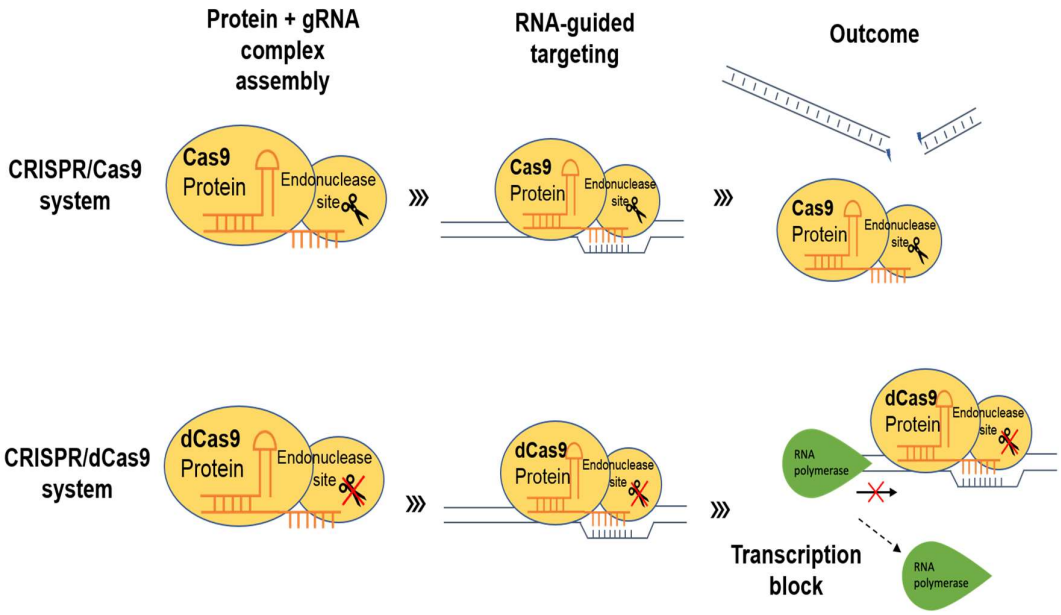

Figure 1. Schematic diagram showing the difference between the CRISPR/Cas9 system and the CRISPR/dCas9 system utilizing Cas9 and deactivated Cas9 (dCas9), respectively.

sgRNA

sgRNA is a combination of crRNA and tracrRNA where they can be linked together with a loop sequence to form the chimeric sgRNA (Uniyal et al., 2019). The two major regions of the gRNA, i.e., (i) the spacer and (ii) scaffold regions, are of particular importance since both regions will form a complex with dCas9 to direct targeted transcriptional regulation. The spacer region contains the crRNA, a 17–20 nt sequence complementary to the promoter region of a target gene, while the scaffold region contains the tracrRNA, which acts as the binding scaffold to bind to the dCas9 protein. To increase the efficiency of CRISPR/dCas9 in transcriptional regulation, the position of the target region is vital. For activation, sgRNA is often designed to target -400 to -50 bp upstream of the translation start site (TSS), while -50 to +300 bp at the TSS is commonly used for repression (Gilbert et al., 2013). The easy modification of spacer region and the synthesis of sgRNAs have made CRISPR/dCas9 into a powerful tool in regulating the expression of transcript levels in planta (Gearing, 2016).

dCas9

dCas9 is a mutated wild type Cas9 (sometimes referred to as dCas9 null mutant). The nuclease domains of Cas9 have been altered by mutating H840A in the HNH domain and D10A in the RuVC1 domain (Qi et al., 2013). The dCas9 is incapable of cleaving DNA but can bind to the target genes guided by sgRNA. Using dCas9 alone, the transcript level of endogenous *TEF1* in *Saccharomyces cerevisiae* was repressed up to 18-fold (Gilbert et al., 2013). The binding of the dCas9 is reversible, which means the expression of a gene can be regulated by dCas9 without modifying the genome permanently. Li et al. (2016) demonstrated that the binding of dCas9/sgRNAs to *yfp* gene can be reversed after removal of dCas9 by washing. The expression of the *yfp* was found to be at its initial level after the removal of dCas9, indicating the usefulness of this feature in the regulation of gene expression.

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Transcriptional effectors

Transcriptional effectors are chimeric proteins that contain DNA-binding domains (Piatek et al., 2015). These effectors can be fused with dCas9 protein to modulate gene expression. If the CRISPR/dCas9 system is paired with a synthetic transcriptional repressor, the expression of the target gene will be repressed (CRISPRi). One such example is Krüppel-associated box (KRAB) (Adli, 2018), which has been commonly used for dCas9-based repression studies (Thakore et al., 2016). Piatek et al. (2015) reported that the transcription activity of *PDS* was remarkably reduced by dCas9:SRDX (the combination of SRDX effector with dCas9 for repression) compared to the control (dCas9 alone). On the other hand, if the CRISPR/dCas9 system is fused with a synthetic activator effector like Herpes simplex viral protein (VP16), transactivation domains of zinc-finger proteins, or transcription activator-like effector (TALE), the transcription of the target gene can be activated (CRISPRa; Casas-Mollano et al., 2020).

Strategies for programmable transcriptional regulation in plants

RNA-guided transcriptional regulation of a gene is a complex process. This process involves the recruitment of activating and repressing transcription factors that are spreading across a large region of the genome (Conaway, 2012). The binding of these regulators to their target DNA sequences can be hampered by epigenetic modifications like histone acetylation and DNA methylation (Kribelbauer et al., 2020). DNA methylation has been shown to disrupt 76% of transcription factors from binding to their target DNA sequences in *Arabidopsis*, indicating that epigenetic modifications could affect the transcription state of genes (O' Malley et al., 2016). The efficiency of repression is also dependent on the host systems. When comparing prokaryotic and eukaryotic cells, the efficiency for dCas9 to repress the expression of the monomeric red fluorescent protein (*mRFP*) in eukaryotes was much lower (20-fold; Gilbert et al., 2013) than prokaryotes (up to 1,000-fold; Qi et al., 2013). Hence, multiple strategies to overcome the low efficiency of transcriptional regulations in eukaryotic cells are indispensable.

Multiple sgRNAs

sgRNAs can be easily manipulated to target several regions of a gene simultaneously. This strategy allows dCas9 to be guided by multiple sgRNAs to bind to different target loci simultaneously (Didovyk et al., 2016). When combining two sgRNAs (each sgRNA has 300-fold repression), Qi et al. (2013) found that the expression of the *mRFP* gene was repressed up to 1,000-fold. The authors also found that the combination of two weaker sgRNAs (each with only 5-fold repression) produced a multiplicative suppression effect up to 20-fold in *E. coli*. This strategy has also been used in plants. In maize, a combination of 2 sgRNAs with a suppressor dCas9 to target the promoter region of maize *phytoene desaturase1* (*PDS1*) showed a 60% reduction of *PDS1* expression, whereas a 2.5-fold increase of *PDS1* expression was detected when using an activator dCas9 (Gentzel et al., 2020). Li et al. (2017) reported that the expression of multiple endogenous genes, *WRKY30*, *RLP23*, and *CDG1*, was enhanced using three sgRNAs. Taken together, these studies showed that multiple sgRNAs could efficiently regulate the expression of a target gene at several regions simultaneously. This is particularly useful for targeting groups of genes in metabolite biosynthetic pathways to enhance the production of desired metabolites. Despite its effectiveness in regulating gene transcription, precautions should be taken to avoid both sgRNAs to compete for the same region.

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Modification of CRISPR/dCas9 components

The CRISPR/dCas9 components, i.e., dCas9 and effectors [such as KRAB (repression) and VP64 (activation) (Figure 2)], can be modified to enhance the efficiency of transcriptional regulation. For example, the modified dCas9, dCas12a (also known as dCpf1), has been found to show better transcriptional repression compared to dCas9 (Zetsche et al., 2015). dCas12a can process a single transcript tandem crRNA array to multiple crRNAs on its own, enabling a more simplified multiplex transcriptional repression compared to dCas9 (Fonfara et al., 2016). Other different designations and modifications of dCas9 to repress gene expression in plant cells are summarized in Table 1.

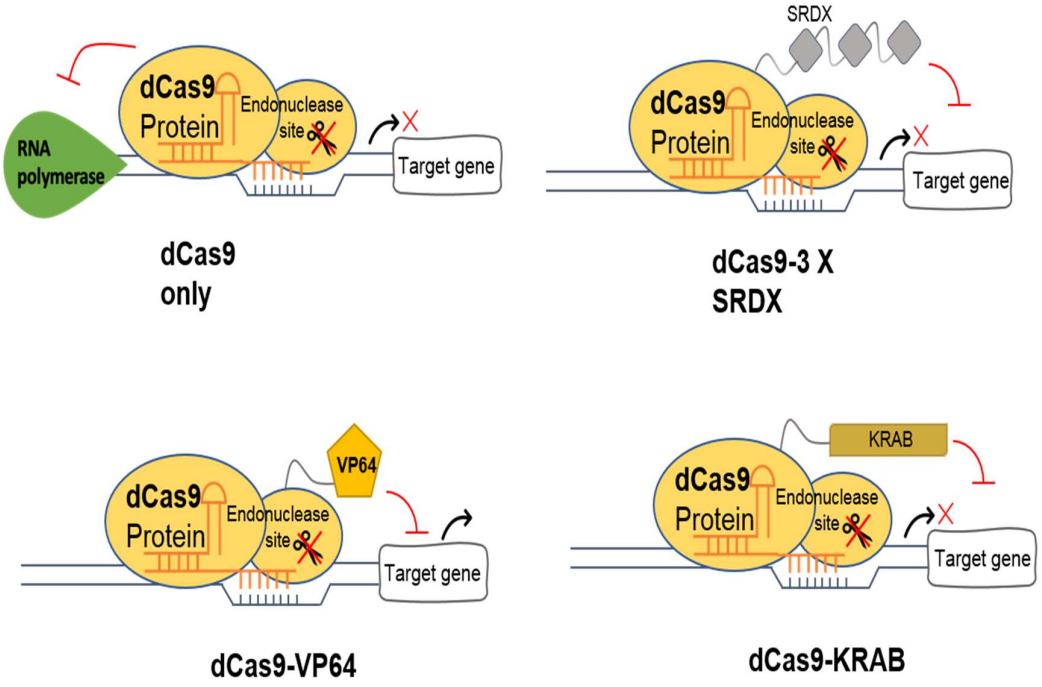

Figure 2. Different types of dCas9 protein with or without the effectors, such as KRAB and VP64.

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Table 1. Modification of repressor for gene repression in plant cells.

Repressor	Plant species	Repression level (%)	Target gene (Number of gRNA)	References
dCas9	*N. benthamiana*	20	PDS (3)	Piatel et al. (2015)
	N. benthamiana	80	pNOS::LUC reporter (3)	Vazquez-Vilar et al. (2016)
dCas9-SRDX	*N. benthamiana*	33	PDS (3)	Piatel et al. (2015)
	N. benthamiana	50	pNOS::LUC reporter (3)	Vazquez-Vilar et al. (2016)
dCas9-BRD	*N. benthamiana*	60	pNOS::LUC reporter (3)	Vazquez-Villa et al. (2016)
dCas9-3xSRDX	*Arabidopsis*	60	CFTS64 (3)	Lowder et al. (2015)
	Arabidopsis	80	miR159A (1)	Lowder et al. (2015)
	Arabidopsis	70	miR159B (2)	Lowder et al. (2015)
dCas9-TALE-SRDX	*Arabidopsis*	-	RD29-LUC (1)	Mahfouz et al. (2012)
	Arabidopsis	-	RD29A (1)	Mahfouz et al. (2012)
dLbCpf1-SRDX	*Arabidopsis*	90	miR159B (1)	Tang et al. (2017)
dAsCpf1-SRDX	*Arabidopsis*	90	miR159B (1)	Tang et al. (2017)

For the effector, an activator-recruiting scaffold can be modified by fusing dCas9 with a tandem array of peptides, known as the SunTag array. This protein scaffold (repeating peptide array) is able to recruit multiple antibody-fusion proteins. By attaching transcriptional factors to the antibodies, dCas9-SunTag can amplify the transcription factor recruitment to the targeted sequence. Through this strategy, Tanenbaum et al. (2014) were able to enhance the activation efficiency of the *CXCR4* and *CDKN1B* genes by recruiting many copies of the VP64 effectors instead of one VP64. Recently, Papikian et al. (2019) examined the effect of the *FWA* gene in *Arabidopsis* flowering by activating this gene using the dCas9-SunTag system. They found that the methylated and silent *FWA* can be upregulated by the dCas9-SunTag system and the effect could be detected up to T2 generation.

Recently, a synergistic activator mediator system (SAM), where an additional sgRNA is engineered through the attachment of a minimal hairpin aptamer to the tetraloop and stem loop 2 of sgRNA, was developed. This aptamer can bind to the dimerized MS2 bacteriophage coat proteins, forming MS2-mediated sgRNA (msgRNA). After fusing with activators, such as p65 and HSF1 transactivation domains, this SAM complex will increase the recruitment of transcription factors and subsequently activate the endogenous gene expression up to 1,000-fold (Konermann et al., 2015). When the SAM strategy was introduced into *Arabidopsis* plants, Park et al. (2017) could trigger the transcriptional activation of the endogenous Anthocyanin pigment 1 (*PAP1*) and vacuolar H+-pyrophosphatase (*AVP1*) genes by 2- to 5-fold, respectively.

CRISPR-Act2.0, a new strategy with a similar design to the SAM, has been recently developed in plants. This strategy uses dCas9-VP64 together with a modified sgRNA which comprises two internal MS2 RNA hairpins. These MS2 RNA hairpins can facilitate the recruitment of additional VP64 via MCPs (Z. Li et al., 2020). It has been shown that the CRISPR-Act2.0 system was better than dCas9-VP64 in activating both protein-coding and non-coding genes in *Arabidopsis* and rice (Lowder et al., 2018).

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Transcriptional regulation toolbox

The delivery of CRISPR/dCas9 constructs into plants to efficiently target multiple genomic loci poses a significant challenge. This is because the designed sgRNAs and dCas9-effector fusion protein are often placed in a single T-DNA (Lowder et al., 2017). To address this shortcoming, a streamlined toolbox utilizing recent cloning methods, such as Golden Gate and Gateway assembly, has been developed. Using this strategy, a pro-dCas9-3X (SRDX) repressor can be easily constructed in 10 days (Lowder et al., 2015). Lowder et al. (2015) found that the expression of *AtCSTF64* in *Arabidopsis* was repressed by 60% using this construct. Another two T-DNA constructs and three sgRNAs were developed together with this toolbox to target *PAP1* and miR319. Lowder et al. (2015) found an increase of 2- to 7-fold and 3- to 7.5-fold of *AtPAP1* and miR319, respectively, which was recorded in the transformed *Arabidopsis* compared to the control. Such targeted gene regulation is expected to allow robust multiplexing in the plant genome.

Plant-specific transcriptional effectors

Plant transcriptional effectors, such as Ethylene Response Factors (ERFs), are an important modulator for gene expression. These transcription factors have been evaluated for developing dCas9-based gene activators in plants. ERFs are important ethylene-signaling regulators for plant defense response against abiotic and biotic stresses. Among the ERFs, the ERF/EREBP family is particularly crucial, as these regulators contain domains with motifs that are unspecified to DNA binding (Azzeme et al., 2017). In the ERF/EREBP family, the SRDX derived from the ERF-related amphiphilic repressor domain (EAR) was found to confer repression activities (Baazim, 2014). It has been used to target the Bs3::uidA (Piatek et al., 2015) and *PDS* genes (Bortesi & Fischer, 2015) in tobacco. Another ERF transcriptional regulator, the EDLL motif, is a strong activation domain that has also been used to activate several genes, such as *PAP1* and *FIS2* in *Arabidopsis* (Lowder et al., 2017; Lowder et al., 2018). However, the dCas9-EDLL with a single sgRNA only showed modest transcriptional activation activities in plant cells (Vazquez-Vilar et al., 2016). Although an attempt was made to fuse the EDLL motif with VP64 to boost the efficiency of transcriptional activation, it failed to work in plant cells (Lowder et al., 2018).

Application of CRISPR/dCas9 in plants

The CRISPR/dCas9 system has emerged as one of the most efficient and cost-saving tools in molecular biology. Besides studying gene function, the CRISPR/dCas9 can also be applied for crop improvements, such as improving resistance/tolerance of plants against biotic and abiotic stresses, regulation of secondary metabolites, and cell imaging (Table 2).

Table 2. Application of CRISPR/dCas9 in plants.

Application	Plant species	Modification	Reference
Live cell chromatin imaging	*N. benthamiana*	dCas9-eGFP	Dreissig et al. (2017)
	N. benthamiana	dCas9-FP	Fujimoto and Matsunaga (2017)
	N. benthamiana	dCas9-MS2-mRuby2	Khosravi et al. (2020)
Transcriptional activation	*Oryza sativa*	dCas9VP64	Lowder et al. (2018)
		LUC/dCas9-TV	Lowder et al. (2017)
		dCas9VP64+ MS2-VP64	Lowder et al. (2018)

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	Arabidopsis	dCas9-TV-6 × His	Li et al. (2017)
		dCas9-MCP-TV	Li et al. (2017)
		dCas9-SunTag	Papikian et al. (2019)
	N. benthamiana	dCas9-TV	Piatek et al. (2015)
		dCas9-VP128	Piatek et al. (2015)
		dCas9-EDLL	Piatek et al. (2015)
Epigenetic manipulation	*Arabidopsis*	dCas9-MS2	Lee et al. (2019)
		dCas9-TET1cd	Gallego-Bartolome´ et al. (2018)
			J. Li et al. (2020)
		dCas9-SunTag	Papikian et al. (2019)
Chromatin topology	*Arabidopsis*	dCas9-PYL1	Morgan et al. (2017)
		dCas9-ABI1	Qi et al. (2013)

Enhancing abiotic stress tolerances in plants

Abiotic stresses, such as drought, flooding, salinity, heavy metals, and heat, have adversely affected the growth and fitness of the plants. Despite extensive research efforts, a feasible and effective method to enhance abiotic stress tolerance in plants is still insignificant. This might be due to the complex regulatory networks, including multifaceted interactions between metabolic, signaling, and regulatory pathways, in plants (Joshi et al., 2016). The use of CRISPR/dCas9 could be beneficial in improving plant stress tolerance. To enhance drought tolerance of *Arabidopsis*, Paixão et al. (2019) introduced a construct, where the dCas9 fused with the *Arabidopsis* histone acetyltransferase 1 (AtHAT1), to activate the abscisic acid (ABA)-responsive element binding protein 1/ABRE binding factor (AREB1/ABF2). The authors observed that the drought-stressed transgenic plants have a higher survival rate and chlorophyll content than control. In a recent study, de Melo et al. (2020) reported that *AREB-1*-activated *Arabidopsis* by CRISPRa showed an improved drought tolerance than wild-type plants. About 2-fold higher relative water content and lower level of malonaldehyde were observed in those transgenic *Arabidopsis* (de Melo et al., 2020). Park et al. (2017) found that a higher accumulation of K^+ and Na^+ ions was detected in transgenic *Arabidopsis* with 2- to 5-fold higher *AVP1* expression than wild-type after activating the transcription of *AVP1* using a redesigned CRISPR/dCas9 activation system. They added a heat-shock factor 1 activation domain and the p65 transactivating subunit of NF-kappa B to the dCas9-VP16.

Improving plant immune against RNA virus

Viruses may affect the growth of their plant hosts, causing significant loss for agricultural sectors (Rubio et al., 2020). Viruses incorporate their genetic material into the plant genomes to reproduce and fabricate the building blocks for new virus particles. Plants defend themselves against virus invasion by activating their RNAi machinery. However, many viruses could inhibit the plant RNAi silencing pathway by releasing a suppressor protein to prevent siRNAs from initiating the process (Burgyán & Havelda, 2011). Since the CRISPR/dCas9 does not have the same silencing pathway as the RNAi, it is more desirable to use such technology to target the viral RNA and disrupt their invasion. Several recent studies have explored the feasibility of CRISPR/dCas9 in improving plant immunity. Khan et al. (2019) showed that the accumulation of cotton leaf curl virus (CLCuV) in tobacco was decreased by 60% using CRISPR/dCas9 compared to the control. It is noteworthy that the efficiency of CRISPR/dCas9 was

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found to be lower than TALE (80%) in inhibiting CLCuV replication. This might be because TALEs are a natural transcription factor that are well adapted in plants (Khan et al., 2019). The multiplexability and ease of designing sgRNAs in the CRISPR/dCas9 system, however, has made this system a valuable tool for inhibiting viral RNA. Besides using dCas9, Cas13a protein from *Leptotrichia shahii* (LshCas13a) or *Leptotrichia wadei* (LwaCas13a), a programmable RNA-guided single-stranded RNA (ssRNA) ribonuclease, has been shown to provide immunity to a bacteriophage in *E. coli* (Abudayyeh et al., 2016, 2017). To reduce turnip mosaic virus (TuMV) in tobacco, Aman et al. (2018) developed a CRISPR/Cas13a construct, which could process pre-crRNA into functional crRNA innately, to target the viral mRNAs. A recombinant TuMV expressing GFP (TuMV-GFP) was then agro-infiltrated into tobacco plants. The authors found that the intensity of GFP-expressing TuMV in tobacco was reduced by up to 50%, indicating successful control over the spread of the viral GFP signal. Recently, Zhang et al. (2019) reprogrammed and expressed the LshCas13a system into rice and tobacco to determine the antivirus effect of the CRISPR/Cas13a system. The authors found that the CRISPR/Cas13a system can degrade genomes of tobacco mosaic virus in tobacco and *Southern rice black-streaked dwarf virus* in rice. Another study, by Zhang et al. (2018), reported a 40-80% reduction of cucumber mosaic virus (CMV) and tobacco mosaic virus (TMV) accumulation in *Nicotiana benthamiana* and *Arabidopsis* using Cas9 from *Francisella novicida* (FnCas9). As demonstrated by Zhang et al. (2018), this FnCas9 could be potentially used to develop FnCas9 immune system conferring RNA virus in plants, as the resistance against CMV can be detected up to T6 generation.

Regulation of secondary metabolites

Plant secondary metabolites are important for plant growth and development. These metabolites have been extensively studied due to their medicinal properties (Gorlenko et al., 2020). To enhance the production of these useful metabolites, several strategies, such as conventional plant breeding and genetic engineering, have been adopted. Plant breeding, however, is a laborious and time-consuming approach as it involves lengthy crossing and backcrossing steps (Lu et al., 2016). On the other hand, manipulation of secondary metabolite biosynthetic pathways at the molecular level has shown promising results but often requires the regulation of multiple key genes simultaneously. The common strategies for secondary metabolite enhancement are (1) overexpressing key genes to ensure sufficient supply of precursors and increase metabolic flux through the target pathway; (2) silencing the key enzyme genes in the competitive pathway of the target metabolite to avoid intermediates being diverted; and (3) overexpressing transcription factors for activation or repression of multiple endogenous key genes simultaneously to enhance the synthesis of the metabolites. The biosynthesis of secondary metabolites is a complex process and often requires simultaneous expression of multiple genes. Multiplexed CRISPR/dCas9 technologies, in which a few sgRNAs or Cas proteins are expressed at once, could be a solution to this. For example, Reis et al. (2019) recently reported that the amount of succinic acid in the CRISPRa-interfered bacteria was about 150-fold higher than the control. They activated 6 succinic acid-related genes by introducing 20 sgRNAs. To date, there are many reports on using CRISPR/dCas9 to enhance metabolite production in microorganisms (Schwartz et al., 2018; Xiong et al., 2020; Yu et al., 2020; Zhan et al., 2020). However, to our knowledge, the use of CRISPR/dCas9 for plant secondary metabolite regulation has not been reported yet, probably due to the complexity of plant secondary metabolisms and inefficient delivery methods.

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Other applications of CRISPR/dCas9

The arrangement and positioning of the genome are vital to the regulation of basic cell functions, such as accurate chromosomal separation in cell division, repair, and replication in DNA, as well as gene expression (Puchta, 2017). To monitor these changes, fluorescent in situ hybridization (FISH) is often used. However, this technique requires one to sacrifice the precious samples, as it involves cell fixation and DNA denaturation steps. On this basis, imaging-based CRISPR/dCas9 could serve as an alternative to FISH. A CRISPR/dCas9-based cell-imaging technique has been developed by Dreissig et al. (2017) through the fusion of two dCas9 orthologs (Sp-dCas9 and Sa-dCas9) with copies of fluorescence proteins to visualize telomeres and to view multiple genomic loci simultaneously in tobacco leaf cells. The authors showed that telomeres are localized in the periphery of interphase nuclei. However, in comparison with FISH, the efficiency of a telomere labeling by dCas9 was 70% (Khosravi et al., 2020). To improve the labeling efficiency of the CRISPR/dCas9 system, various orthologues of Sp-Cas9, including St1-Cas9 and Sa-Cas9, can be recruited in combination with modified sgRNAs with RNA aptamer MS2/PP7 insertions that bind to a fluorescent coat protein (Khosravi et al., 2020). Using this method, the dynamics of telomeres and centromeres in living plant cells can be traced.

As previously mentioned, dCas9 can be fused with epigenetic regulatory factors to modulate chromatin modifications. The current approaches for the studies of epigenetic regulation are often tedious and costly since these techniques require intensive labor and pose a risk of unspecific targeting. Since dCas9 can be fused with DNA methylase or demethylase to regulate the level of DNA methylation, the CRISPR/dCas9 technology could be used to understand the epigenetic regulation. For example, dCas9 fused with mammalian acetyltransferase (p300) was used to target the promoter region of *IL1RN*, *MYOD1* (*MYOD*), and *POU5F1/OCT4* (*OCT4*) genes to enhance the histone H3 acetylation at lysine 27 (Hilton et al., 2015). Lee et al. (2019) developed a CRISPR/dCas9 construct containing MS2 epigenetic regulator (dCas9-MS2VP64) to target the flowering time regulator *FT* gene in *Arabidopsis*. They found that about 65% of CRISPRa-interfered *Arabidopsis* showed a moderate shift in flowering time compared to the wild type (Lee et al., 2019).

Challenges and issues of CRISPR/dCas9

Although there is the excitement of using CRISPR/dCas9 to facilitate advanced crop improvement, there are some challenges. Deriving from the CRISPR system, CRISPR/dCas9 shares the same limitations with the CRISPR system, i.e., off-target effects. This unwanted event happens when a sequence homologous to the desired sequence or a similar sequence with some mismatches is targeted. To reduce the possibility of off-target effects, several free online prediction tools have been developed to assist researchers in designing sgRNA. These online tools are CRISPOR (Concordet & Haeussler, 2018) and CCTop (Stemmer et al., 2015). Another strategy by changing the structure of Cas9 to decrease its ability to bind to partly mismatched gRNAs could probably reduce the off-target effects. However, this strategy may have little significance for the CRISPR/dCas9, since the frequency of off-target effects for this technique is lower than the conventional CRISPR. In fact, the CRISPR/dCas9 system only active if dCas9 binds to a 350 bp long sequence in proximity to the promoter of the target gene. A mismatch of one base pair in CRISPR/dCas9 decreases its performance, whereas multiple mismatches have made it inactive. Moreover, the off-target repression is not permanent and can be reversed by removing the dCas9 from the wrong target. In comparison, the CRISPR system edits DNA sequences regardless of the location in the

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genome which can produce a higher frequency of off-target effects than CRISPR/dCas9.

In comparison with the RNAi technique, one of the challenges faced by CRISPR/dCas9 is the requirement of PAM sequences for the dCas9 to be precisely position at the target gene. The PAM sequence determines the specificity of the CRISPR/dCas9 system. However, it may restrict the application of CRISPR/dCas9 if there are limited PAM sites in a genome. As described above, the recent discovery of Cas proteins that can recognize different PAM sequences could certainly help to expand the versatility of the CRISPR/dCas9 technology. Since CRISPR/dCas9 has lesser off-target effects and higher on-target efficiency than RNAi (Larson et al., 2013), it is probably a better alternative tool for gene functional studies. The RNAi, however, is still suitable for high-throughput screening since less information is needed for the siRNA design.

Another limitation of CRISPR/dCas9 is that this system does not exist naturally in plants, meaning that CRISPR/dCas9 components like Cas proteins must be introduced into plant cells. The introduction of these components can be time-consuming (Boettcher & Michael, 2015) and sometimes requires codon optimization steps if the dCas9 is from different origins (Choudhary et al., 2016). The inefficient delivery of CRISPR/dCas9 into the plants remains a major barrier to realizing the potential of this technology. This is mainly due to the recalcitrance of plant tissues/cells and the inability of plant tissues/cells to regenerate. Hence, a novel delivery method such as direct delivery of CRISPR/dCas9 constructs into plant apical meristem to circumvent tissue culture is desirable.

GM plants are highly affected by regulations and society's perception (Menz et al., 2020). For example, only 11.9% of the population in China had a positive view of GM foods, whereas 41.4% and 46.7% had neutral and negative views, respectively (Cui & Shoemaker, 2018). Similar to the CRISPR-edited plants, each country has its perspectives on whether CRISPR/dCas9 like CRISPRi should be equally treated as traditional GM plants. The Chinese and EU governments have concluded that every organism "whose genome constitution has been changed by using genetic engineering technology" (State Council of China, 2001), to be applicable for CRISPR, is a GMO that needs strong regulation and pre-release authorization (Directive 2001/18). In contrast, the United States has exempted CRISPR-edited crops from the GMO regulations, particularly if the CRISPR system is removed from the modified organism after the editing is executed (Globus & Qimron, 2018), for instance, evidence of foreign DNA was not found on a CRISPR-edited mushroom (Waltz, 2016). Since the CRISPR/dCas9 does not change the DNA of an organism, it could probably be treated differently in the authorization process (Globus & Qimron, 2018). The CRISPR/dCas9-interfered plants, however, may still require approval from the authority since they contain foreign dCas9. While it is still debatable, it is most likely that CRISPR/dCas9-interfered plants will be declared as GMOs. More investigations are thus essential to clarify whether consumption of CRISPRi-interfered crops/fruits containing dCas9 could be harmful to humans.

Conclusions

CRISPR/dCas9 is an innovative and increasingly popular technology for functional genomics and crop improvement. It could be used to regulate the transcription of targeted genes without altering their sequence. With an improved CRISPR/dCas9 system through modifications of dCas9 proteins and effectors as well as the use of multiple sgRNAs or a transcriptional regulation toolbox, this technology could

efficiently regulate the expression of multiple target genes simultaneously. The CRISPR/dCas9 system has now been used to enhance the resistance or tolerance of plants to biotic and abiotic stress, regulate secondary metabolite biosynthesis, and track chromatin dynamics in live cells. Despite its importance, several limitations of the CRISPR/dCas9 must be addressed to fully exploit the approach for crop improvement. Public concern for possible hazards due to the consumption of a CRISPR/dCas9-interfered crop and the question of whether plants made this way are GMOs demand further investigation. While there is much to be explored, CRISPR/dCas9 is undeniably a powerful tool that will flourish into a mature technology to support genome engineering requirements in crop improvement programs.

Conflicts of Interest

No potential conflict of interest was reported by the authors.

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