Applications of CRISPR/dCas9-Based Systems in Plant Sciences

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 gene functional studies. Deriving from the CRISPR/Cas9 system, both systems consist of a catalytically dead Cas9 (dCas9), a transcriptional effector and a single guide RNA (sgRNA). This type of dCas9 is incapable to cleave 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.

1. The CRISPR/Cas System: Discovery and Principle

CRISPR was first discovered unintentionally in the genome of *Escherichia coli* by Ishino et al. [1] while sequencing the *iap* gene. The authors found an unusual series of tandemly repetitive 29 nucleotide (nt) DNA sequences interspaced with 32-nt spacer sequences downstream of the gene [1]. They have no clue about the biological function of these repeats since it lacks sequence homology to other known sequences at that time. Later, while sequencing numerous fragments in the genome of *Haloferax mediterranei*, Mojica et al. [2] discovered long tandem repeats. This marked the first time that direct repeats were detected in archaea. Mojica et al. [3] classified such interspaced repeat sequence as short regularly spaced repeats (SRSRs). To avoid naming confusion in future studies, Jansen et al. [4] 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. [4].

In 2005, it was discovered that the spacers within CRISPRs were derived from invading phages and plasmids [5,6,7]. 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 [8,9,10,11]. These authors found that new spacer sequences from the infecting phage are acquired into bacterial CRISPR array.

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, Type I system uses Cas3 to cleave target DNA [12], whereas Type III system utilizes Cas10 with the aid of polymerase and repeat-associated mysterious proteins (RAMPs) to cleave RNA and DNA [13]. In comparison, 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) [14]. The mature dual tracrRNA:crRNAs form a complex with a Cas9 protein that can recognize the protospacer adjacent motif (PAM) and cleave specifically at 3 bp before the PAM site of the double-stranded DNA [15]. In 2012, the research group led by Emmanuelle Charpentier and Jennifer A. Doudna published a landmark paper [16], detailing the application of class II CRISPR/Cas9 system for gene editing. This ground-breaking discovery has earned these two scientists the 2020 Nobel Prize in Chemistry.

2. 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. In addition, studying gene function, the CRISPR/dCas9 can also be applied for plant improvements, such as improving resistance/tolerance of plants against biotic and abiotic stresses, regulation of secondary metabolites and cell imaging.

2.1. 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 lacking. This might be due to the complex regulatory networks, including multifaceted interactions between metabolic, signalling and regulatory pathways, in plants [133]. The use of CRISPR/dCas9 could be beneficial in improving plant stress tolerance. To enhance drought tolerance of *Arabidopsis*, Paixão et al. [134] 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 the control. Recently, de Melo et al. [135] reported that *AREB-1*-activated *Arabidopsis* by CRISPRa showed an improved drought tolerance compared with wild type plants. A 2-fold higher relative water content and lower level of malonaldehyde were observed in those transgenic *Arabidopsis* [135]. Park et al. [120] found that a higher accumulation of K^+ and Na^+ ions was detected in transgenic *Arabidopsis* with 2- to 5-fold higher *AVP1* expression and improved tolerance to drought stress compared with wild type after activating the transcription of *AVP1* using a redesigned CRISPR/dCas9 activation system. They redesigned their CRISPR/dCas9 activation system by adding a heat-shock factor 1 activation domain and the p65 transactivating subunit of NF-kappa B to the dCas9-VP16.

2.2. Improving Plant Immunity against RNA Virus

Viruses may affect the growth of their plant hosts, causing a significant loss for the agricultural sectors [136]. Viruses incorporate their genetic material into the plant genomes to reproduce and fabricate the building blocks for new virus particles. Plants defence 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 [137]. 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 been carried out to explore the feasibility of CRISPR/dCas9 in improving plant immunity after the reports on inhibiting virus *in vivo* using variants of Cas protein, namely Cas9 from *Francisella novicida* (FnCas9) and the Cas effector from *Leptotrichia shahii* (LshCas13a) or *Leptotrichia wadei* (LwaCas13a) [138,139]. Zhang et al. [140] reported a 40–80% reduction of cucumber mosaic virus (CMV) and tobacco mosaic virus (TMV) accumulation in *N. benthamiana* and *Arabidopsis* using FnCas9 and discovered that FnCas9 inhibits the virus in a CRISPRi fashion. The repression of CMV virus was not affected even without the endonuclease's activity of FnCas9, indicating that the RNA-virus inhibition by FnCas9 is due to its RNA-binding capability. As demonstrated by Zhang et al. [140], this CRISPR/dCas9 system could be potentially used to develop stable transgenic RNA-virus resistant plants since the resistance against CMV in *Arabidopsis* can be detected up to T6 generation. Another study by Khan et al. [141] showed that the accumulation of Cotton Leaf Curl Virus (CLCuV) in tobacco was decreased by 60% using CRISPR/dCas9 compared to control. It is noteworthy that the efficiency of CRISPR/dCas9 was 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 [141]. However, the multiplexability and the ease of designing sgRNAs in the CRISPR/dCas9 system is still an alternative for the inhibition of viral RNA. To reduce turnip mosaic virus (TuMV) in tobacco, Aman et al. [142] developed a CRISPR/dCas9 construct containing Cas13a, 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 up to 50%, indicating the successful control over the spread of the viral GFP signal.

2.3. 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 [143]. 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 [144]. 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 the 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 for this.

For example, Reis et al. [145] recently reported that the amount of succinic acid in the CRISPRa-interfered bacteria was about 150-fold higher than 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 [146,147,148,149]. 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.

2.4. Other Applications of CRISPR/dCas9

Genome structure is crucially important 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 [150]. 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 the FISH method. A CRISPR/dCas9-based cell imaging technique has been developed by Dreissig et al. [127] 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% [129]. 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 an RNA aptamer MS2/PP7 insertions that bind to a fluorescent coat protein [129]. Using this method, the dynamics of telomeres and centromeres in living plant cells can be traced.

The epigenetic regulatory mechanisms are essential for plant development and adaptation to the environment. As previously mentioned, dCas9 can be fused with epigenetic regulatory factors to modulate chromatin modifications. This makes the CRISPR-based epigenetic regulators a promising tool to investigate the relationships between specific phenotypes and chromatin features. However, the current approaches for the studies of epigenetic regulation are often tedious and costly since these techniques require intensive labor work 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 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 [151]. Lee et al. [121] 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 [121]. Although most examples described here were developed in model species, we envisage that epigenetic versions of well-established alleles conferring favorable traits will be established in crop species.